SUSCEPTIBILITY OF DIABETICS WITH SUPEROXIDE DISMUTASE GENE 2 POLYMORPHISM TO VASCULAR COMPLICATIONS

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BONAFIDE CERTIFICATE

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ABBREVATIONS

AGEs	–	Advanced glycation end products.
PKC	–	protein kinase C
12/15-LO	-	12/15-lipoxygenase
ROS	-	reactive oxygen species
FFA	-	Free fatty acids.
LDL	-	Low density lipoproteins.
SOD	–	Superoxide dismutase.
MnSOD	-	Manganese superoxide dismutase.
Ala16Val	–	Alanine 16 valine.
C/T	-	Cytosine/thymine.
MTS	-	mitochondrial targeting sequence.
RAGE	-	Receptor for AGE.
(NF)-κB	-	nuclear factor kappa B.
VCAM-1	-	Vascular Cell Adhesion Molecule 1
VEGF	-	Vascular endothelial growth factor.
DAG	–	Diacyl glycerol.
SHP-1	-	Src homology-2 domain–containing phosphatase-1.
MAPK	-	Mitogen activated protein kinase.
PDGF	-	platelet-derived growth factor .
GFAT	-	glutamine:fructose 6-phosphate amidotransferase .
UDP	-	GlcNAc- UDP-*N*Acetylglucosamine.
PAI-1	–	Platelet activator inhibitor -1 .
TGF-_1	-	Transforming growth factor-1.
VSMC	–	vascular smooth muscle cells.
PARP	-	polyADPribose
eNOS	–	endothelial nitric oxide synthase.
NO	–	Nitric oxide.
AT1R	-	Angiotension receptor.
MI	–	Myocarial infarction.
GPX-1	-	glutathione peroxidase .
DM	–	Diabetes Mellitus.
HYT	–	Hypertension.
ROS	-	Reactive oxygen species

INDEX

INTRODUCTION

Diabetes mellitus is the most important cause for vascular diseases of heart and brain. Increased cardiovascular disease risk among diabetic patients from various racial and ethnic groups have been found by different studies[1]. One of the major causes for death among diabetic patients includes myocardial infarction and other cardiovascular diseases which account for about 50% of all diabetes mortalities, and much morbidity[2]. Many factors including genetic factors are involved in the pathophysiology of cardiovascular disease in diabetes. The combining factor in the development of diabetic complications is oxidative stress[3]. Oxidized LDL results in atherosclerotic plaque [4] formation that leads to vascular complications. It is a fact that among diabetics some develop vascular complications but not seen in others.This may be due to presence of some gene polymorphism in those developing complications. Gene polymorphisms in antioxidant enzymes like superoxide dismutase and catalase in diabetes has been reported [5]. This polymorphism results in decrease in level and activity of antioxidant enzymes which leads on to oxidative stress. This increase in oxidative stress can lead on to atherothrombotic complications. A polymorphism in superoxide dismutase 2 gene which changes the secondary structure of

mitochondrial targeting sequence of the manganese superoxide dismutase enzyme is seen. This polymorphism is present in exon 2 of the gene where normal GCT is mutated to GTT. This results in creation of a restriction site(rs 4880) as well as change of aminoacid from alanine to valine at 16th position. This enzyme otherwise called as manganese superoxide dismutase is present inside mitochondria. This polymorphism in mitochondrial targeting sequence of the enzyme causes impaired targeting of the SOD 2 enzyme resulting in reduced activity of the enzyme inside mitochondria. A study has shown that individuals with this polymorphism had thickened carotid wall making them prone for cardiac complications. [6] . This polymorphism causes defective targeting of superoxide dismutase to mitochondria where it is required to combat oxidative stress, which can lead to development of oxidized LDL and accelerated foam cell formation in atherosclerosis[7].

This polymorphism is also supposed to be related with cancers of breast [8], lung [9], Parkinsons disease[10] , anterior uveitis[11]. Milan Flekac et al showed a positive association between mitochondrial superoxide dismutase gene polymorphism and vascular complications in diabetes.

This study is done to find out if this polymorphism is associated with cardiovascular complications in diabetes, and to correlate it with superoxide dismutase activity.

REVIEW OF LITERATURE

Diabetes Mellitus is a heterogeneous disease characterized by defective synthesis and/or secretion of insulin, as well as by resistance of the peripheral tissues to the hormone activity.

Globally around 100 million people suffer from diabetes. By 2025, it is supposed to rise to 300 million. As per 2011 statistics, there are about 61 million diabetic patients in India[12]. The mortality rate of diabetes in India is just less than a million in 2011[13].

Many etiological factors are considered to be responsible for diabetic micro- and macroangiopathy where persistent hyperglycemia plays the leading part[14].There is a 3- to 8- fold increased risk of cardiovascular disease among patients with diabetes and impaired glucose tolerance. About one third of patients with acute myocardial infarction have diabetes and another one third have impairment of glucose tolerance[15].

Atherosclerosis is the main risk factor for cardiac problem in diabetes mellitus[16]. There are various risk factors for atherosclerosis, some of which are unmodifiable like age, male sex, socioeconomic status and some of which are modifiable like Insulin resistance &

hyperglycemia, cigarette smoking, alcoholism, obesity, oxidative stress, hypertension.

Oxidative stress is the main cause of atherosclerosis and other diseases such as cancers, rheumatic arthritis, haematological and neurodegenerative disorders and diabetes mellitus [17] .The free radical concentration in the body increases either due to elevated production or deficent removal due to deficiency of scavenging enzymes. Oxidative stress causes diabetes as well as its micro and macrovascular diseases due to diabetes.[18].

PATHOPHYSIOLOGY OF DIABETIC VASCULAR DISEASE

The key factors contributing to atherosclerosis and plaque rupture in diabetes [19] include

1. Endothelial dysfunction and vascular smooth muscle cell dysfunction.

2. Dyslipidemia.

3. Thrombogenic state.

All these abnormalities occur in diabetes due to oxidative stress caused by hyperglycemia and insulin resistance.

One of the major mechanisms for development of diabetic vascular complications is oxidative stress by superoxide anion. Increased superoxide production in diabetes occurs due to hyperglycemia inside cells or high free fatty acid levels in endothelial cells. High FFA level in diabetes is due to insulin resistance causing increased FFA release from adipocyte which move in to endothelial cells.

Hyperglycemia and high FFA in diabetes causes tissue and endothelial cell injury by various ways.

(1) activation of polyol pathway due to hyperglycemia.

(2) damage by elevated production of advanced glycation end products.

(3) Stimulation of protein kinase C enzyme

(4) Augmented formation of hexosamines[20].

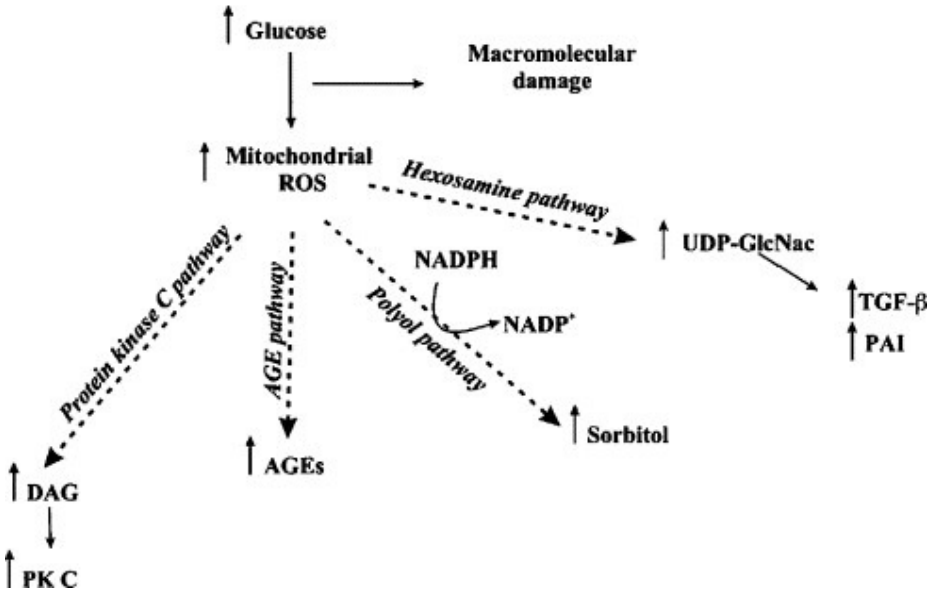
(5) Stimulation of the 12/15-lipoxygenase (12/15-LO) enzyme[21] .

These pathways are commonly stimulated by increased synthesis of superoxide inside mitochondria[22]

This elevated synthesis of superoxide radical occurs due to hyperglycemia and high FFA levels which in turn activate all the mechanisms of complications as shown in figure 1.

Figure 1

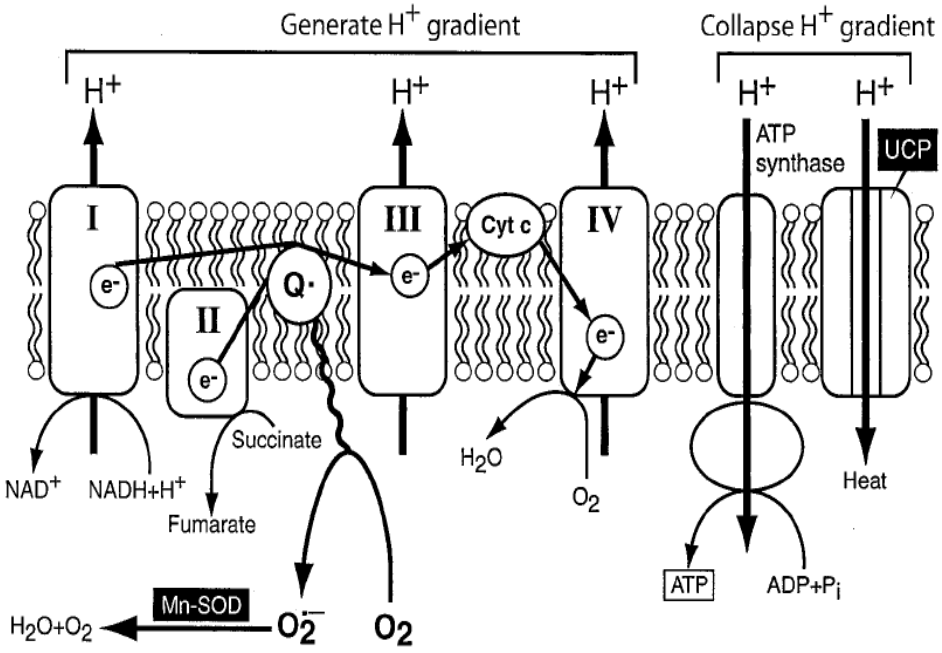
Hyperglycemia in diabetes causing oxidative stress and activating damaging pathways of complications.



The high levels of glucose and FFA in diabetic cells and endothelial cells result in more glucose and FFA getting burnt in citric acid cycle. This causes increased transfer of reducing equivalents in the respiratory chain. This results in increase in the electrical potential until up to a level when there is inhibition of third complex of electron transport chain[23]. This results in reversal of transfer of electrons from third complex to

coenzyme Q which in turn gives it to oxygen producing superoxideanion ,(Fig. 2). This superoxide is dismutated by manganese superoxide dismutase enzyme present in mitochondria. So patients with ala 16 val SOD2 gene polymorphism have decreased mitochondrial superoxide dismutase leading on to increased concentration of superoxide which activates the damaging pathways.

Figure 2



Hyperglycemia-induced production of superoxide by the mitochondrial electron transport chain.

Microvascular complications in diabetes are also due to intracellular hyperglycemia. In addition to hyperglycemia, macrovascular complications are due to elevated fatty acid level caused by insulin

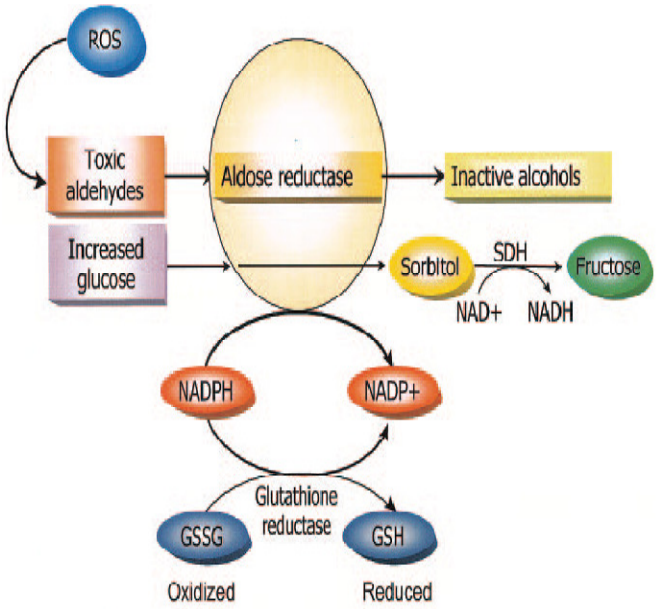
resistance. As only specific cell types are affected by generalized hyperglycemia it seems that affected cells fail to check the glucose entry during hyperglycemia . When glucose level is high, vascular endothelial cells exhibit no significant reduction in glucose transport rate, resulting in intracellular hyperglycemia.[24].

Activation of polyol pathway

This mechanism was described in the 1966 *Science* paper I.The harmful aldehydes produced in tissues is converted to harmless alcohol by aldose reductase. However when there is hyperglycemia, there is increased production of sorbitol from glucose by this enzyme, which utilizes its coenzyme NADPH [25] . Fig. 3.

Figure 3

Activation of polyol pathway.



NADPH is essential for synthesis of reduced form of glutathione, which is needed for the enzyme glutathione peroxidase involved in reducing free radicals. As NADPH is used up by this polyol pathway, tissues fail to defend free radical damage. The reactive oxygen species then damages endothelial cells which may act as initiating factor for development of atherosclerosis.

Damage by advanced glycation end products

Whenever there is hyperglycemia, there is increased production of advanced glycation end products. These are produced by spontaneous reaction between glucose and proteins.

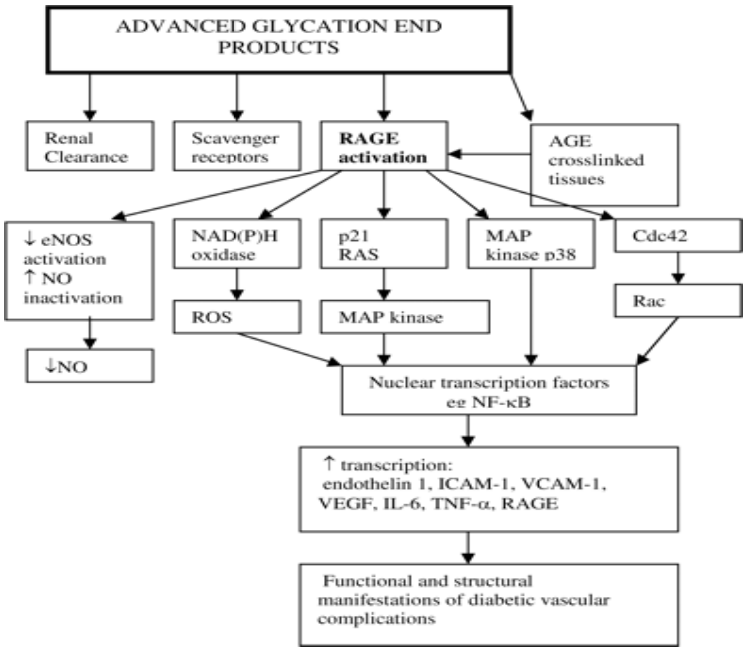
These seem to injure cells by various ways.

The foremost one is the glycation of proteins inside the cells which act as transcription factors and coregulators.[26]

The advanced glycation end products disrupt the communication between cells and extracellular matrix by altering the proteins forming the matrix. This results in malfunction of endothelial cells with failure of normal protective function [33] .

Another way by which advanced glycation end products exert its effect include the attack of plasma proteins like albumin by the precursors of advanced glycation end products. The receptors for advanced glycation end products in endothelial cells get stimulated when they interact with AGE precursors. This stimulation increases the synthesis of mediators and growth factors involved in atherosclerotic plaque formation. Stimulation of (NF)-κB, resulting in various alteration in gene expression occurs by the interaction of advanced glycation end products with its receptors.[27].

This interaction also stimulates the transcription of genes coding for thrombomodulin and other coagulation factors in endothelial cells. It also induces the transcription of cell adhesion molecules and various growth factors which bring about adhesion of inflammatory molecules on to endothelial cells and raised permeability of blood vessels.[28]See figure 4.



By all these ways advanced glycation end products bring about atherothrombotic changes in blood vessels.

Increased Protein Kinase C Activation

High levels of ROS inhibit the glycolytic enzyme glyceraldhyde - 3-phosphate dehydrogenase (GAPDH) which in turn leads to enhanced production of DAG from triose phosphate. This DAG causes excessive activation of several PKC isoforms[29]. The binding of advanced glycation end products with their receptors can result in augmentation of signaling pathway with stimulation of isoenzymes of protein kinase C[30]. This results in phosphorylation and activation of pathogenic mechanisms.

It includes increased production of SHP-1 which is a tyrosine phosphatase enzme.

It also inhibits the signal transduction through platelet derived growth factor receptor by causing dephosphorylation. This may have adverse effect on pericytes around endothelium, causing their death[31].

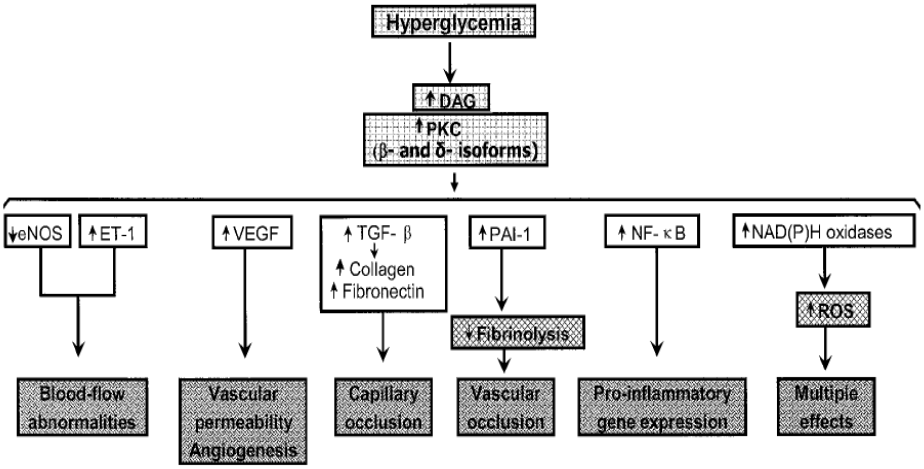
Similarly increased fatty acid oxidation plays a significant role in initiating atherosclerosis by damaging endothelial cells and also cardiac cells which causes cardiomyopathy.

Increased protein kinase C furthermore results in stimulation of nuclear factor kappa B which induces many genes involved in inflammation causing damage to blood vessels.

The consequences of increased blood glucose concentration and stimulation of protein kinase C[32,] is shown in fig 5.

Figure 5.

Consequences of hyperglycemia induced activation of protein kinase



Augmented formation of hexosamines

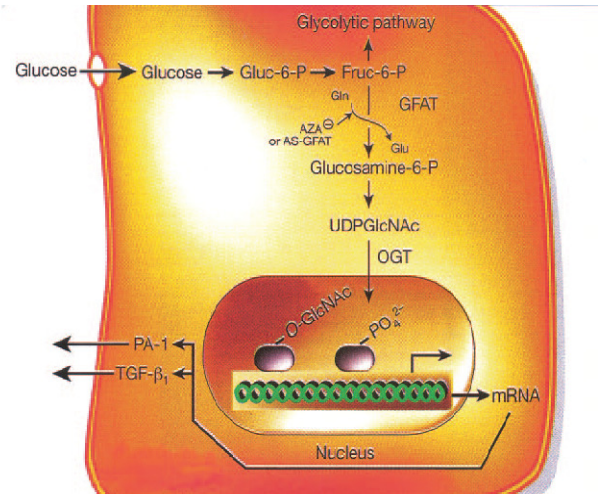
High blood glucose concentration and increased fatty acid level seen in diabetes results in high levels of fructose-6-phosphate. These then enter in to the path of aminosugar formation. Both hyperglycemia and insulin resistance induced excess fatty acid oxidation , increase the flux of fructose -6-phophate into the hexosamine pathway. Fructose 6-phosphate is transformed to glucosamine phosphate by amidotransferase which transfers amino group from glutamine to fructose-6-phosphate. This is subsequently transformed to UDP-*N*Acetylglucosamine.

UDP- GlcNAc acts as a source of NAcetylglucosamine which is added to serine residues present in various proteins, especially those involved in transcription of genes [33]. This especially causes stimulation of genes

concerned with atherosclerotic plaque formation. In smooth muscle cells of blood vessels, N-glycosylation of transcription factor Sp1 results in switching on of promoter of plasminogen activator inhibitor -1 . Within endothelial cells, this causes stimulation of plasminogen activator inhibitor -1 and transforming growth factor-1, which are involved in pathogenesis of atherosclerosis seen in figure 6.

Figure 6.

Hyperglycemia increases flux through the hexosamine pathway which plays a role in atherothrombosis



12/15-lipoxygenase (12/15-LO) pathway

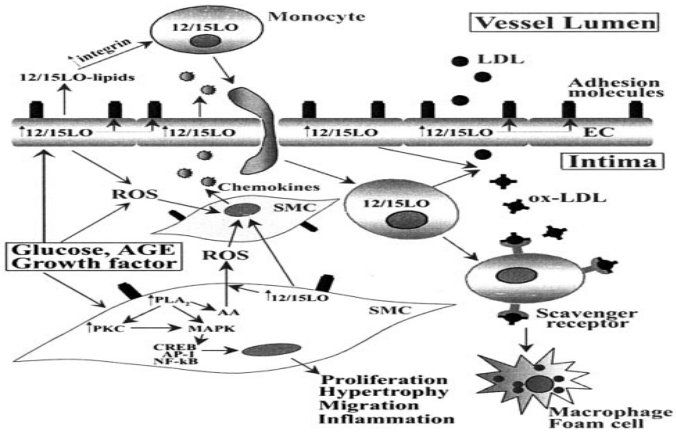
Hyperglycemia promotes the production of lipoxygenase enzymes, both 12 and 15 isoenzymes. The low density lipoproteins get oxidized by these enzymes and this oxidized lipoprotein is highly atherogenic than normal low density lipoproteins. This oxidation mostly occurs in macrophages. Such type of pathogenic oxidation of low density lipoproteins are found in endothelial cells as well as smooth muscle cells[34].

12-lipoxgenase causes stimulation of transcription of cell adhesion molecules like VCAM-1 over the surface of endothelial cells, which results in binding of endothelium to inflammatory cells including macrophages.

It as well causes increased transcription of proteins present in extracellular matrix, inflammatory chemokines which attract inflammatory mediators to endothelium. Moreover, it has been shown to cause augmented growth of vascular smooth muscle cells. See figure 7.

Figure 7.

Actions of 12/15-LO in the vessel wall



Certain studies concluded that, if superoxide radical was destroyed by manganese superoxide dismutase enzyme, the high blood glucose concentration was unable to stimulate the damaging mechanisms[35] . This has been verified by conducting research in mice which were genetically altered to produce increased concentration of manganese superoxide dismutase enzyme.

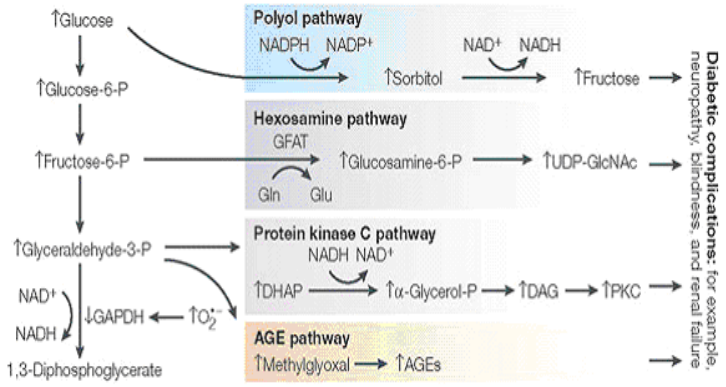
Augmented superoxide synthesis inside mitochondria by increased blood glucose level stimulates the various detrimental mechanisms by inactivating glyceraldheyde -3-phosphate dehydrogenase enzyme.

When the glyceraldheyde -3-phosphate dehydrogenase enzyme becomes inactivated by increased mitochondrial superoxide production, there is accumulation of previous products in reaction of glycolysis.

Each accumulated intermediate of glycolysis pathway causes stimulation of each one of the detrimental pathways. figure 8.

Figure 8.

Hyperglycemia causing activation of damaging pathways by inhibition of GAPDH(Glyceraldehyde-3-phosphate dehydrogenase).



1. Increase in glyceraldehydes 3 phosphate activates AGE pathway by forming methylglyoxal from glyceraldehyde-3 phosphate.

2. Formation of diacylglycerol from glyceraldehyde-3 phosphate activates PKC pathway.

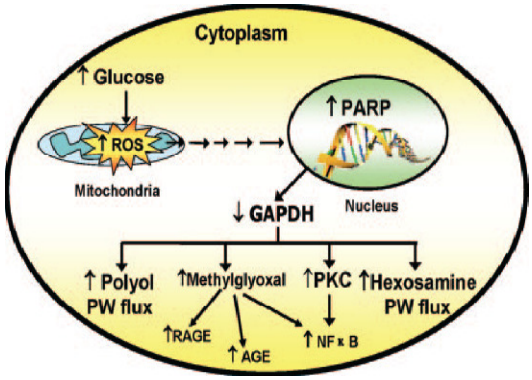
3. Increase in fructose -6 phosphate results in formation of UDP–*N*acetylglucosamine by the enzyme GFAT activating hexosamine pathway.

4. Activation of the polyol pathway , due to reduction of glucose by aldose reductase ,which utilizes NADPH,therby causing depletion of NADPH.

Augmented superoxide synthesis inside mitochondria by increased blood glucose level inactivates glyceraldheyde -3-phosphate dehydrogenase enzyme by causing stimulation of poly(ADPribose) polymerase[36] as seen in figure 9.

Figure 9

Hyperglycemia-induced mitochondrial superoxide production inhibits GAPDH by activating poly(ADPribose) polymerase.



Superoxide production by hyperglycemia and fatty acid oxidation cause DNA strand breaks which activates poly(ADPribose) polymerase PARP. This PARP then splits NAD in to nicotinic acid and ADP-ribose which modifies and inhibits GAPDH activity.

Studies show that both manganese superoxide dismutase enzyme and uncoupling protein-1 can stop these mechanisms from occurring [50].

They have also shown that death of podocytes and damage to endothelial cells are stopped by use of inhibitors of PARP.

These inhibitors of PARP apoptosis, improved the symptoms of neuron damage and kidney damage[37] .

In addition to above processes, the augmented free radical production caused inactivation of the important enzymes needed to prevent atherogenosis .They include prostocyclin synthase and endothelial nitric oxide synthase[38,] .

Catalytic antioxidants

Salvemini D et al found that use of analogues of either superoxide dismutase or catalase resulted in stoppage of inhibition of the enzyme prostacyclin synthase by superoxide radical in diabetic animals[39].

Hyperglycemic memory

It has been observed that even though the blood glucose level had been in control for many months, there was still injury occurring to various cells and tissues. This is known as “hyperglycemic memory”. The reason can be attributed to epigenetic modifications done by free radical like superoxide anion. When the level of superoxide anion or methylglyoxal derived from it was reduced, it completely stopped the generation of these epigenetic modifications. This has been revealed by various studies[40]. So the increased risk of patients for complications with SOD2 polymorphism can be deduced from this.

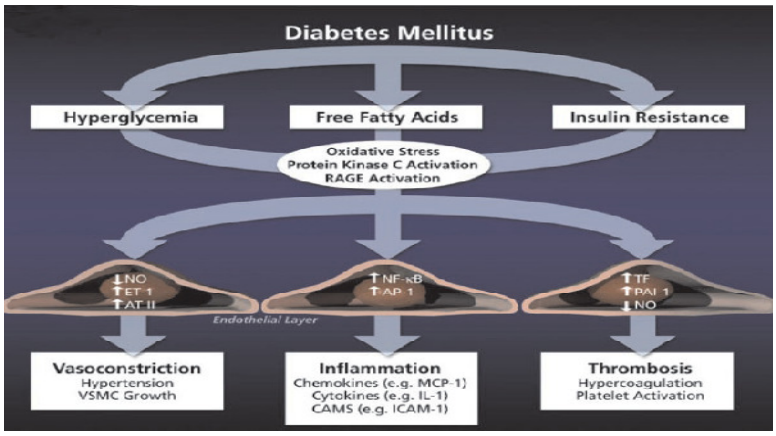
Studies by Ballinger et al and Semenkovich CF et al in rat model showed that there is increased formation of atherosclerosis in rats having insufficient mitochondrial manganese superoxide dismutase enzyme[41,].

In transgenic mouse models of cardiovascular disease, favorable effects of overexpression of antioxidant enzymes had been observed. The reasonable drug of choice for ameliorating the oxidative stress caused by hyperglycemia would be analogue of manganese superoxide dismutase for these experiments.[56.]

As previously pointed out, the key factors contributing to atherosclerotic lesions in diabetes include endothelial dysfunction, dyslipidemia, thrombogenic state. figure 10.

Figure 10

Mechanism of atherosclerosis in diabetes mellitus



Endothelial dysfunction;

Endothelial cells produce nitric oxide (NO) constitutively to preserve vascular homeostasis . The role of nitric oxide is to produce vasodilatation by causing relaxation of VSMC.

Nitric oxide also prevents the movement and hyperplasia of VSMC. Moreover, it inhibits the binding of endothelial cells to inflammatory cells and platelets. Nitric oxide also prevents stimulation of nuclear factor kappa B , which is involved in production of inflammatory mediators contributing to atherosclerosis [42]. All these are caused by activation of signal transduction pathways by nitric oxide. So nitric oxide acts as a defensive mechanism against the endogenous injury of endothelium and atherosclerosis'

Nitric oxide levels are decrease in hyperglycemia, which may be due to impaired NO production or increased degradation of NO.

Impaired nitric oxide production can be due to following reasons.

As previously explained, hyperglycemia and free fatty acids lead to increased superoxide generation which activates PKC pathway which inturn produces superoxide by activating NADPH oxidase. This superoxide inactivates NO and converts it into peroxynitrate [43]. The formed peroxynitrate inhibits tetrahydrobiopterin ,which acts as the coenzyme for the nitric oxide synthase enzyme, thereby inhibiting nitric oxide synthase[44].

Activation of hexosamine pathway and PKC pathway by superoxide anion results in inhibition of the enzyme Akt kinase,involved in activation of nitric oxide synthase enzyme. So this causes reduced synthesis of nitric oxide[45].

Normally, DMA dimethylaminohydrolase is recquired to degrade ADMA(Asymmetric Dimethyl Arginine), which inhibits nitric oxide synthase enzyme. But this enzyme is inhibited by the free radical superoxide anion, resulting in accumulation of ADMA . This accumulated ADMA inhibits the enzyme nitric oxide synthase[46].

Insulin plays an important role in relaxation of blood vessels [47], by stimulating nitric oxide synthase enzyme through PI-3kinase pathway. The impaired insulin sensitivity or reduced insulin level seen in diabetes ,thus causes reduced production of nitric oxide . But the

stimulation of MAPK by insulin remains unaltered ,which causes various atherothrombotic changes by altering endothelin levels.

VASCULAR SMOOTH MUSCLE DYSFUNCTION

As already explained, the relaxation of blood vessels by nitric oxide is reduced in diabetes mellitus. There has also been seen increased movement and proliferation of VSMC outside the endothelium, resulting in aggravation of atherosclerotic lesion[48]. There is moreover augmented destruction of VSMC in atherosclerotic lesion, which accounts for easy disintegration of plaque. Elevated synthesis of MMP by inflammatory mediators result in degradation of collagen found in plaque[49].

DYSLIPIDEMIA

One of the precipitating factors for development of atherosclerosis is high lipid levels. Most of the diabetic patients have abnormal lipid levels.

The type of low density lipoprotein seen in diabetes is abnormal in size and density. It is smaller and denser than normal low density lipoprotein, which allows them to pass through the blood vessel wall and get adhered to it. From there, they are vulnerable to the attack by free radicals[50].

This oxidatively modified low density lipoproteins are seen by leukocytes as alien particles. This results in engulfment of these particles by white blood cells, initiating the formation of foam cells. These foam cells cause propagation of macrophages, VSMC [51] , resulting in development of atherosclerosis.

Also, there is persistence of low density lipoproteins due to addition of glucose to it, resulting in development of atherosclerosis.

But the defensive effect of high density lipoprotein is lost, when glucose molecules are added, as it causes early degradation of high density lipoproteins.[52]

Hypertriglyceridemia can also lead to increased production of the small, dense form of LDL and to decreased HDL transport of cholesterol back to the liver in diabetes[53].

Hypertriglyceridemia occurs in diabetes because of reduced activation of LPL, due to low insulin level which causes reduced triglyceride breakdown from VLDL and chylomicrons. And also low levels of insulin results in loss of inhibition of HSL, leading on to high FFA concentration.

THROMBOTIC STATE

The common final pathology of MI is the disintegration of fibrous cap leading on to coagulation and blockage of large blood vessel.. Diabetes is a thrombotic state because there is hyperactivity of platelets and transcription of glycoproteins ,which are involved in binding of VWF to platelets[54]. There is decreased platelet-derived NO, due to increased superoxide anion formation associated with high intracellular platelet glucose concentration and PKC activity [55] . Platelet conformation is altered in diabetes with release of mediators due to impairment of calcium homeostasis [56].

In diabetes, there is elevation of levels of various clotting factors and reduction of various anti clotting factors.[57]. Moreover, there is increased synthesis of PAI-1, which results in elevated risk of thrombosis complicating plaque rupture.

Superoxide dismutases

Superoxide dismutases, increase the rate of degradation of superoxide. The superoxide is converted to O_2 and H_2O_2.

$$2O^{2-} + 2H^{+} \longrightarrow O_2 + H_2O_2$$

Superoxide dismutases (SODs) are the enzymes which play a primary role against oxidative stress.

Action mechanism

It has 2 reactions.

The first reaction is the one whereby superoxide is converted to oxygen by oxidation.

In the second reaction, superoxide is reduced to hydrogen peroxide.[58]

O_2°- + Mn(III)SOD →O2 + Mn(II)SOD -------- oxidative reaction

O_2°- + 2H+ + Mn(II)SOD → H2O2 + Mn(III)SOD--- reductive reaction

3 isoenzymes of superoxide dismutase are present. They include

1. Superoxide dismutase 1 also called CuZn superoxide dismutase,present mainly inside cytoplasm. It is also localized inside nucleus and lysosomes,having a molecular weight of 32,000 Da.

2. Superoxide dismutase 2 also called as Mn superoxide dismutase ,present inside mitochondria[59].

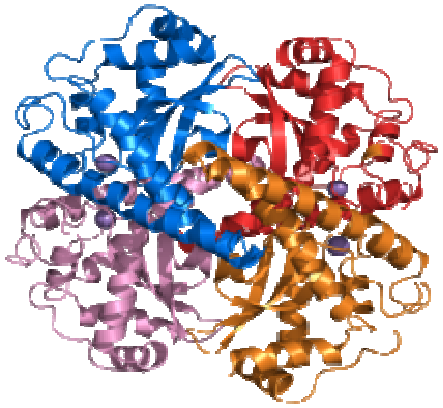
3. Superoxide dismutase 3 ,also called as extracellular SOD having copper and zinc as its cofactor. It is mainly seen in blood ,also

present in CSF and ascitic fluid. It has four similar subunits ,with molecular mass of 135,000 daltons.[60]

Manganese superoxide dismutase has 4 similar subunits,with each monomer weighing about 23,000 daltons (fig 11)[61]. Each monomer is supposed to have 2 parts, an all alpha amino terminal part and an alpha or beta carboxy terminal part.SOD2 has been shown to play a important part in scavenging mitochondrial superoxide ,playing a major role in preventing formation of cancer [10] and in defending hyberbaric oxygen stimulated lung injury.

Figure 11

Structure of MnSOD

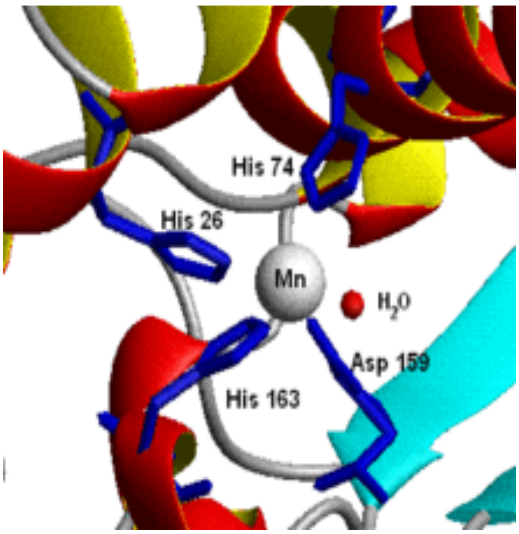


Specific areas of superoxide dismutase 2 enzyme seem similar in different species of animals, thus projecting them to be necessary for

their catalytic mechanism. Many of them ,especially,histidine at 26th and 81st position and glutamine at 67th and 171th position of Eschericia coli act as ligands for metal ions. Some of them act as entry point to active site[62] . The manganese forms a coordination complex with 5 groups, 4 of them being side chains of proteins and the last being either H_2O or OH^- (fig 12)

Figure 12

ACTIVE SITE OF MANGANESE SUPEROXIDE DISMUTASE ENZYME



Structure of manganese superoxide dismutase gene

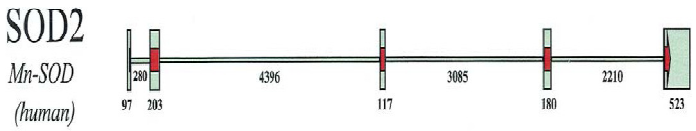
The human superoxide dismutase 1 gene is found to present in long arm of 21st chromosome[63] . The human superoxide dismutase 3 is found

to be situated in 4th chromosome [64]. The human superoxide dismutase 2 is found to be situated in 25th position of long arm of 6th chromosome[65] .

There are about five sequences of exons and four intronic sequences seen in the manganese superoxide dismutase gene. (see Fig. 13).

Figure 13

Structure of MnSOD gene.



Particularly in humans,there is absence of promoter sequences like TATA box and CAAT box ,but areas rich in guanine,cytosine residues with binding sites for activator protein 2 and sp1 is seen[66]. This enzyme is produced along with signaling sequence which directs it mitochondria after its synthesis from mitochondria [67]. Once the enzyme reaches mitochondria ,the targeting sequence is removed resulting in formation of active protein[68].

The polymorphism analysed in this study is present in the region coding for mitochondrial targeting sequence of SOD2 gene [10] . At 1183rd position of the gene, normally there is cytosine as GCT codon which codes for alanine. It causes proper conformation of the MTS in to an alpha helix, which properly targets the enzyme to mitochondria. Transversion of cytosine to thymine produces GTT codon which codes for valine instead of normally coded alanine. This causes improper folding of the MTS in to beta sheet structure, with failure of targeting in to mitochondria,which also gets destroyed by proteasome. [10] .The distribution of alanine coding codon among white population seems to be about 50%, whereas in oriental population ,it is very low of about 13-30%.,[69] . Individuals can have alanine in both the alleles,or valine in both the alleles,or alanine in one allele,and valine in another allele [11]. So this is a functional polymorphism which is found to be common in this gene. Some studies have shown that messenger RNA of valine coding gene is speedily destroyed (Sutton et al., 2005). Polymorphism in the promoter area of the superoxide dismutase gene has also been proposed to decrease the transcription of this gene,with resulting decrease in enyme levels in cancer cell lines. (Xu et al., 1999b)[70].

Another mutation in promoter area of this gene is observed,where cytosine replaces thymine,causing altered interaction of activator protein -2,with reduced expression of the enzyme. (Xu et al., 1999b).

Toxic superoxide

Superoxide causes multiple pathological changes within the cell.

SOD protects the cell from superoxide toxicity. One of them include conversion of nitric oxide to harmful peroxynitrate. Minute levels of superoxide itself will inhibit TCA cycle enzymes and electron transport chain. The superoxide anion radical (O_2^-) spontaneously dismutes to O_2 and hydrogen peroxide (H_2O_2) quite rapidly (~10^5 $M^{-1}s^{-1}$ at pH 7). SOD is necessary because superoxide reacts with sensitive and critical cellular targets. For example, it reacts the NO radical, and makes toxic peroxynitrite. The dismutation rate is second order with respect to initial superoxide concentration.it also causes accumulation of harmful free iron. [106].

Regulation of superoxide dismutase concentration

It is very important to maintain optimum conentration of supaeroxide dismutase to combat free radicals,which are constantly being produced in the body.

This enzyme seems to be regulated at various levels.

First is at the level of transcription, where numerous transcription factors are involved in its regulation.

The most important one is the nuclear factor kappa B which responds rapildly to tissue injury. There has been found response elements for this transcription factor in both introns and promoter in all 3 isoenzymes of superoxide dismutase.

Superoxide dismutase 1 cannot be stimulated rapidly because it is synthesised continuosly . But promotor of this gene responds to nuclear factorκB through phosphotidylinosital-3 kinase pathway and increases its expression.

The expression of superoxide dismutase 2 is increased quickly by harmful radiation,iflammatory mediators and certain interferons like IFN-γ.

AT-II, iflammatory mediators, NO have regulatory role over transcription of superoxide dismutase 3.

Second important transcription factor is specificity protein 1,which has zinc finger motif through which it binds nuclear material ,especially

in guanine,cytosine rich regions and brings about increased expression of all 3 superoxide dismutases.

Another transcription factor includes activator protein 1 which has got fos as well as jun DNA binding domains through which they activate the genes for superoxide dismutases. Ironiclly,the level of this activator protein is reduced by excess of the manganese superoxide dismutase enzyme.

Another transcription factor is activating protein 2,which can interact with different transcription factors. When this factor bind to promoter region of superoxide dismutase 2 gene, it prevents stimulation of expression by specificity protein 1,thereby reducing the transcription of this gene.

Regulation by epigenetic mechanism

Changes in manganese superoxide dismutase in breast cancer is attributed to repression of the gene by epigenetic changes such as methylation. One explanation for the association of atherosclerosis with superoxide dismutase 3 is due to hypomethylation.

Modifications after transcription

Stabilty of messenger RNA and ineraction of the messenger RNA to specific proteins are also important in translation of this trnscripts. Alu region seen in 3' untranslated region of the mRNA seems to be targetted by micro RNA which controls its expression. A RNA binding protein needs to be bound to this 3' untranslated region to increase its expression.

Therapeutic use of superoxide dismutase

It has been proved a safe therapeutic agent in animal as well as human studies. It has been used to treat complications of radiation following carcinoma of breast. It has also shown improvement in burns patients,systemic lupus erythematosus,herpes simplex and fibrosis due to liver damage by hepatitis C and various other diseases.[71]

It has been considered a potential target of gene therapy. Especially, gene for manganese superoxide dismutase complexed with either liposome or with plasmid reduced irradiation related damage in various carcinomas.

It has also proved benefical in kidney,testes reducing reperfusion injury following ischemia in rats. Also protection protection against myocardial infarction and restenosis has been shown.

So, in future, it has got potential role in traeatment of various diseases.

Diseases associated with altered MnSOD level

Apart from complications in diabetes,changes in manganese superoxide dismutase concentration was found to be linked with degenerative diseases like Parkinson,s disease,CMT disease and DMD[72]. Reduced levels of the enzyme has been found in various kinds of cancer[73] .

Other diseases associated with MnSOD gene polymorphism

MnSOD polymorphism and diabetic retinopathy and nephropathy

Retinopathy in diabetes is due to damage to vascular endothelium by inflammation and high levels of VEGF in vitreous causing blood vessel proliferation. This occurs due to increased superoxide causing activation of protein kinase C and other damaging pathways leading on to inflammation of retinal blood vessels and elevated VEGF production. This increase in superoxide level may be due to reduced degradation of superoxide by MnSOD Ala16Val polymorphism[74]. Studies in japan,korea have shown linkage between this polymorphism and nephrophathic changes in diabetes patients[75] .

MnSOD polymorphism and asthma

A study showed that patients with Ala genotype have a greater risk of asthma than those with Val-Val genotype [76].The reason is higher MnSOD activity causes increased production of cytotoxic hydroxyl radicals and hydrogen peroxide which when acted upon by myeloperoxidase produces hypochlorous acid that causes cellular damage in adjacent epithelial cells

The *MnSOD −9 Val-Ala/Ala-Ala* genotypes were at greater risks of asthma than those carrying the *Val-Val* genotype.

MnSOD polymorphism and schizophrenia

One study has observed positive association between this mutation with schizophrenia and propensity of development of tardive dyskinesia among these patients [77]. The reason that neurons getting particularly damaged include reduced concentration of catalase,glutathione peroxidase,increased oxygen utilization and increased rate of metabolism, making them prone for oxidative stress.

Another reason they are highly prone for oxidative stress is that the neuronal membrane is mainly made of polyunsaturated fatty acids,which rapidly get attacked by reactive oxygen species. This peroxidation

disrupts many vital functions like ion channels and communication systems.[78]

MnSOD polymorphism and anterior uveitis

One study has observed association between anterior uveitis and polymorphism in manganese superoxide dismutase gene,where there is transversion of guanine to adenine at position 47. it particularly rises the propensity of the diseas in persons having HLA-B-27[79].

MnSOD polymorphism and breast cancer

In contrary to above said observations that alanine allele is protective for various diseases, it has been observed in other studies that,alanine genotype with raised manganese superoxide dismutase is associated with tumours of colon [80],breast[81],lung[82] etc.

MnSOD polymorphism was also studied in diseases like rheumatoid arthritis,bechet's disease, ovarian cancer,prostate cancer.

Polymorphism in superoxide dismutase 1 and superoxide dismutase 3

About 11 mutations in Cu/Zn SOD or superoxide dismutase 1 has been found to be associated with amyotrophic lateral sclerosis in a study performed by Daniel R.Rosen et al.[83]

A study conducted in preterm infants found that a polymorphism at 8192287 restriction site of extracellular superoxide dismutase showed a defensive effect against development of intraventricular hemorrhage. [84]

Also a polymorphism in Cu/Zn superoxide dismutase showed a protective effect against development of retinopathy of prematurity,respiratory distress syndrome and intraventricular hemorrhage. Similarly,another mutation in superoxide dismutase 2 showed protective effect against development of retinopathy of prematurity,respiratory distress syndrome and intraventricular hemorrhage but showed risk of developing bipolar disorder among preterm infants.

A study conducted in extracellular superoxide dismutase among diabetic individuals observed that threonine was more frequent than alanine at 40^{th} position. Also insulin sensitivity to insulin was low among diabetic individuals with threonine compared to alanine[85].

AIMS AND OBJECTIVES

Diabetes is a common cause of cardiovascular disease especially in developing countries. It is a major contributor to mortality among patients suffering from non-insulin dependant diabetes. Genetic factors make the diabetic patients prone for cardiovascular complications.The knowledge of genetic factors of diabetic cardiovascular disease may help in explaining the molecular basis of this disorder and in designing prevention and treatment methods. Literature evidences point to the role of MnSOD gene polymorphism in the causation of cardiovascular complications in diabetes.

The aim of this study is

1. To analyse if there is increased susceptibility of diabetic patients with MnSOD gene polymorphism to cardiovascular complications.

   This is done by finding out the distribution of MnSOD gene polymorphism among type 2 diabetic patients with and without cardiovascular disease and in healthy controls.

2. To assess if this MnSOD polymorphism causes reduced SOD activity.

MATERIALS AND METHODS

This is a case-control study conducted after obtaining ethical committee clearance. This study was conducted in the time interval of February 2012- August 2012 at Madras medical college and Rajiv Gandhi government general hospital.

Study population:

CASES:

About 60 diabetic patients with more than five years duration attending cardiology ward, diabetology ward and outpatient department were included in the study after obtaining consent and were categorised into

Group 1A: 30 (14 males, 16 females) type 2 diabetic patients with cardiovascular disease and

Group 1B: 30 (11 males, 19 females) type 2 diabetic patients without cardiovascular disease.

The diagnosis is based on clinical history, fasting plasma glucose levels and ECG findings (pattern of myocardial infarction in case of Group 1A and normal pattern in case of Group 1B)

Exclusion criteria:

Patients less than 30 years of age and less than 5 years of duration of diabetes.

CONTROLS:(Group 2)

30 individuals attending master health check- up were selected as controls.

Age, sex and other confounding factors like smoking, alcoholism were matched .

Sample collection:

Blood samples:

Blood was collected after an overnight fast of 8-12 hrs. About 4 mL of blood was drawn from the cubital vein of the subjects. 2 mL was transferred into a EDTA tubes and another 2ml transferred in to serum tube.

Serum was used for superoxide dismutase activity. EDTA tube was centrifuged at 2000 rpm for twenty minutes to get buffy coat for DNA

extraction and the plasma was used for glucose and lipid profile estimation.

DNA extraction was done on the same day and extracted DNA stored at -20°C.

METHODS:

BUFFY COAT SEPARATION

Buffy coat was separated by centrifugation of EDTA tubes at 2000 revolutions per minute for 20 minutes. Buffy coat was transferred to 2mL eppendorf and was used for DNA extraction. Plasma separated was used for glucose and lipid profile .

Serum superoxide dismutase activity ,plasma triglycerides,plasma HDL,plasma cholesterol,plasma glucose, were analysed by using kits in ERBA semiautoanalyser. Low density lipoprotein cholesterol (LDL-c) was calculated using Friedwald's formula.

DNA EXTRACTION BY MODIFIED HIGH SALT METHOD[86]

RBC Lysis:

- 400µL of buffy coat in a 2mL eppendorf was mixed with 1.6mL of 0.17M ammonium chloride and mixed by inversion until red cells were lysed for about 10 minutes
- The cells were centrifuged at 4000rpm for 10minutes.
- The white cell pellet was washed with 800µL of 0.17M ammonium chloride solution. The procedure was repeated till a clear white cell pellet is obtained.

WBC Lysis

- To the pellet 500 µL of TKM I solution was added. It is centrifuged at 10,000rpm for 10minutes.

Nuclear Lysis

- The supernatant was discarded. To the pellet 500 µL of TKM II solution was added. To 300 µL of 6M Nacl and 50 µL of 10% SDS was added.
- Mixed well (vortex), Centrifuged at 10,000 rpm for 10 minutes.
- The supernatant was saved and transferred to 1.5mL eppendorf.

DNA Precipitation

- To the supernatant double the volume of 100% ethanol was added.
- The sample was stored at -20◦C for 1 hour.
- Then centrifugation at 4◦C and 10,000 rpm was done for 20minutes using refrigerated centrifuge.
- The supernatant was discarded. To this 500 µL of 70% ethanol was added. The pellet was mixed and centrifuged at 10,000 rpm for 10minutes at 4◦C.
- Pellet was air dried after throwing the supernatant.

Storage

30 µL of LTE buffer was added to the pellet and the extracted DNA was stored at -20◦C for future use.

Identification

Extracted DNA was identified by 0.9% agarose gel electrophoresis with a constant voltage of 7V/ cm and comparison with a known molecular weight 1kb DNA ladder. **Figure:14**

Figure 14

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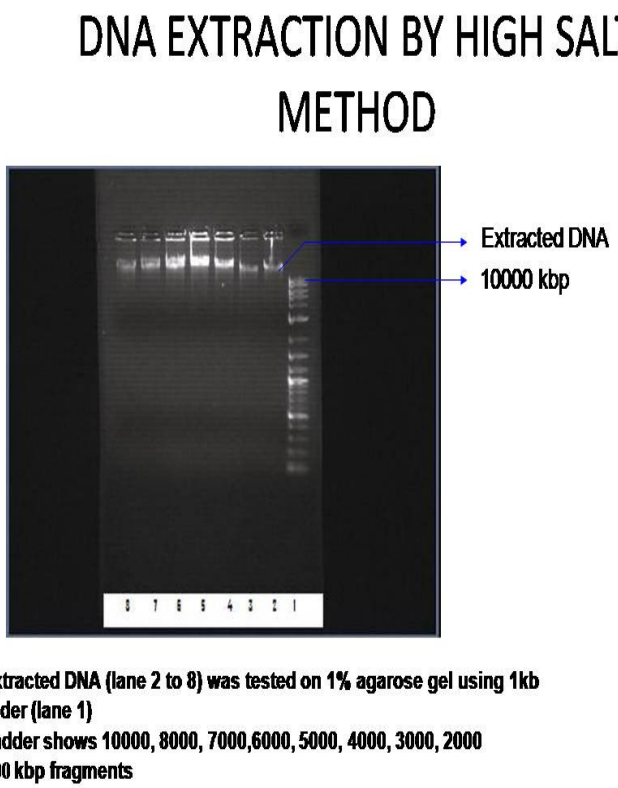

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Concentration of extracted DNA:

- Concentration of extracted DNA was estimated using UV spectrophotometer at 260 nm.

- Concentration was calculated using the formula :

1 OD is equivalent to 50 µg/mL.

Conc. of DNA = absorbance X 50 µg/mL X dilution factor

= y X 50 X 100 ng/µL

- Purity of extracted DNA was assessed by 260nm/280nm.

POLYMERASE CHAIN REACTION

SOD2 gene was amplified using,

- Forward primer – 5'GCTGTGCTTTCTCGTCTTCAG 3' and
- Reverse primer – 5'TGGTACTTCTCCTCGGTGACG3'

Primer Reconstitution

Primers were supplied in lyophilized form.

Autoclaved distilled water was used to prepare 100 × concentrations i.e. 10times the molecular weight of primer was the volume of water required to prepare 100 × concentration which is 100µmolar solution.

- From this stock solution 10 × concentration was prepared as the working solution for PCR.

MASTER MIX:

- Helini master mix in the following composition was used..
- Reaction buffer consisted of Tris Hcl -10mM at pH 8.3
- KCl - 50mM
- MgCl2 - 2mM acts as catalyst.

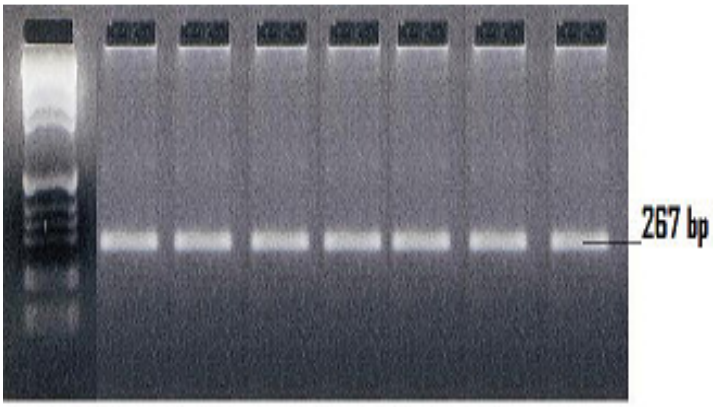
- dNTP's were used in a concentration of 200μM each.
- Taq polymerase in a concentration of 1.25 U.
- Primers were used in a concentration of 5 pmol and DNA was used in a concentration of 200ng.
- The following components were mixed in a 25 μL PCR mixture.
- PCR master mix – 10.5 μL
- 5X red dye _ 2
- Forward, Reverse primer – 1 μL each
- DNA – 2μL
- Distilled H_2O – 8.5 μL
- Total – 25 μL
- Amplification was carried out in an Mc Genei thermal cycler with the following cycling conditions.
- Initial denaturation – 94^0 C -5min
- 30 cycles of

- Denaturation – 94^0C – 1 min
- Annealing - 60^0C – 1min
- Extension -72^0C – 1min
- Final extension at 72^0C - 10 min.

Amplified product 267 bp by PCR was identified by 2.5% agarose gel electrophoresis by comparison with a known 100bp DNA ladder.

Figure 15

It shows 267 bp PCR product on 2.5% agarose gel. First lane shows 100 bp ladder.



AGAROSE GEL ELECTROPHORESIS

- PCR product was run on agarose gel in a 30 mL agarose cast as follows: 750mg of agarose is weighed and dissolved in 30mL of TAE buffer with a pH of 8.0.

- It was microwaved for 60 secs, cooled and 2.5 µL of ethidium bromide (10mg/mL) was added. It was poured into a cast and allowed to solidify for 15 min before it was kept in the electrophoresis tank.

- 8 µL of PCR product was loaded onto wells and 4 µL of 100bp DNA ladder was loaded onto single well as a marker. It was electrophoresed at 7V/cm for 45min and visualized under UV illumination.

RESTRICTION DIGESTION

The PCR product was digested by 5U BsaWI restriction enzyme by incubating overnight at 60 degree Celsius .

Principle of BsaWI enzyme digestion

- C allele in GCT codon does not have a restriction site and hence will yield a 267 bp fragment.
- If T allele is present (GTT codon), a restriction site was created and so the PCR product gets cleaved to give 184bp and 83 bp fragments.
- Heterozygous individuals (CT genotype) will have 267 bp, 184 bp and 83 bp fragments.
- Analysis was done using 100 bp DNA ladder.

Restriction digestion is carried out by the following protocol.

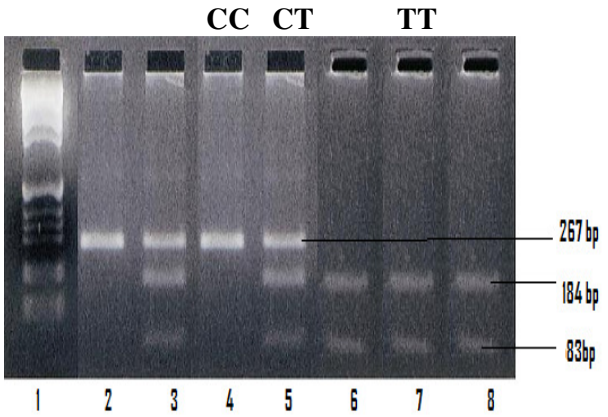
- Distilled water - 2.5µL
- 10X NE Buffer 4 - 5µL

- BsaWI enzyme - 0.5µL(5U)
- PCR product -17µL
- Total -25µL

After overnight incubation at 60 degree celsius the digested products were run in agarose gel electrophoresis using 2.5% LE(low endosmosis) agarose and visualized by UV illumination. Figure16.

Figure 16

Agarose gel electrophoresis of restriction digestion fragments after digestion with BsaW1 enzyme. Lane 1-molecular weight marker(100-bp ladder);lanes 2,4-homozygote CC genotype; lanes 3,5-heterozygote CT genotype; lanes 6,7,8 –homozygote TT genotype



Estimation of Fasting plasma glucose:

Method: Glucose oxidase peroxidase (GOD/POD), Enzymatic method

Principle:

$$\text{Glucose} + O_2 \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{Phenolic compound} + \text{4aminoantipyrine} \xrightarrow{\text{Peroxidase}} \text{Pink coloured complex} + 2H_2O$$

The intensity of pink coloured compound is comparative to level of glucose and analysed at a wavelength of 505nm.

Reagent composition

Glucose oxidase-20000IU/L

Peroxidase-3250IU/L

4-Aminoantipyrine-0.52 mmol/L

4-Hydroxybenzoic acid-10mmol/L

Phosphate buffer-110mmol/L

Glucose standard-100mg/dl.

Procedure:

To 1 ml of working reagent, 10 µL of plasma was added and placed in incubation at for 15 min at a temperature of 37°C .

Reference range: Fasting plasma glucose - → 70 - 100 mg/dL.

Estimation of Plasma Total Cholesterol

Method

Cholesterol Esterase – Cholesterol Oxidase

Kit used

Autospan of Span Diagnostics Ltd.

Principle

Cholesterol ester is hydrolysed by cholesterol esterase to cholesterol.the cholesterol is then acted upon by oxidase to form cholestenone and hydrogen peroxide. The hydrogen peroxide combines phenol and aminoantipyrine to give red coloured compound and the measurement was done using 500nm.

Reagents

Reagent 1 (Enzymes / Chromogen)

It contained all the enzymes and 4.aminoantipyrine.

Reagent 1A (Buffer)

It contained sodium cholate,pipes buffer and phenol.

Standard -200mg/dL

Cholesterol 2g/L

Procedure

To 1 mL of the reconstituted reagent, 10 µL of plasma was added and reading was taken after 5 mins of incubation at 37^{o} C.

Reference Values

Cholesterol : 150-260 mg /dL.

Estimation of Plasma Triglyceride

Method

Enzymatic Colorimetric method

Kit Used

Autopak of Bayer Diagnostics

Principle

The triglycerides in the sample was hydrolysed by lipoprotein lipase to givr glycerol and fatty acid. The glycerol was then acted upon by kinase to form glycerol-3-phosphate,which is converted to hydrogen peroxide and DHAP by oxidase. This hydrogen peroxide ,then combines 4-aminoantipyrine and ADPS to form red colour dye.

Measurement was made at 546nm.

Reagents

Reagent 1 (Enzymes / Chromogen)

It contained all the enzymes and ATP and 4-aminoantipyrine.

Reagent 1A (Buffer)

Pipes buffer. pH 7.50	50mmol/L
ADPS	1mmol/L
Magnesium salt	15 mmol/L

Standard (Triglycerides 200mg / dL)

Glycerol (Trig.Equivalent) 2g/L

Procedure

To 1 mL of the reconstituted reagent 10 µL of plasma is added and read at 546nm after incubation at 37^{o}C for 5mins.

Reference Range

Males 60- 165 mg/dL

Females 40- 140 mg/dL

Estimation of HDL Cholesterol

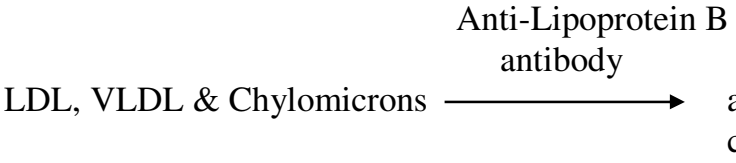
Method Immunoinhibition

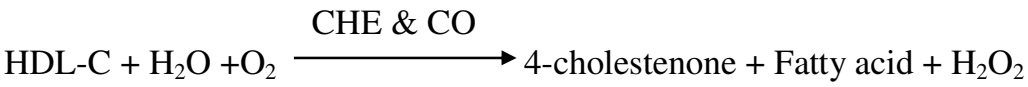
Kit used Erba XL System Packs

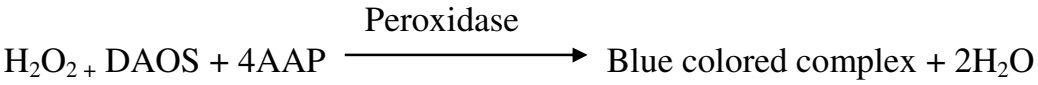
Principle

When anti human antibody **to** β-lipoprotein was added,it formed complex with all lipoproteins except HDL.

This HDL only can react with cholesterol oxidase and esterase resulting in production of hydrogen peroxide. This hydrogen peroxide then combines with F-DAOS and aminoantipyrine to form blue coloured compound and the measurement taken at 593nm.







Reagents

Reagent 1

Goods buffer pH 7.0	30.0mmol/L
4-AAP	0.9mmol/L
POD	2400U/L
Ascorbate oxidase	2700U/L

Antihuman β lipoprotein antibody

Reagent 2

Goods buffer, pH – 7.0	30.0mmol/L
CHE	4000U/L
CO	20000U/L
F-DAOS	0.8mmol/L

Calibrator

HDL-C	56.5mg/dL

Procedure

Reagent 1 & 2 are mixed in the ratio of 3:1 or 1 bottle of reagent 1 was mixed with 1 bottle of reagent 2 and placed in the auto analyser .

Assay type	:	2 point
Primary wavelength nm	:	600, Secondary wavelength nm : 700
R-1 volume	:	270, R-2 volume : 90
Reaction direction	:	increasing, Sample volume : 3 µL
Calibration	:	straight

Reference Values

Adult male : 35.3 – 79.5 mg /dL

Adult female : 42.0 – 88.0 mg / dL

VLDL and LDL Cholesterol

These parameters were calculated using Friedwald's formula given below:

LDL-C = TC-(HDL-C+ VLDL-C)

VLDL-C = TGL/5

Estimation of serum superoxide dismutase activity;

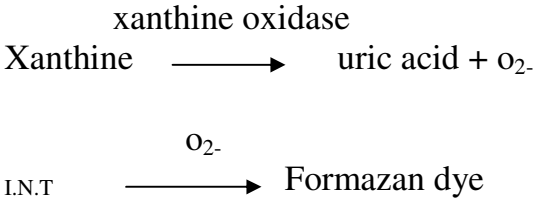
METHOD; Xanthine method, enzymatic method

Kit used- **Fortress Diagnostics Limited**

Principle ;

Xanthine oxidase in the reagent oxidizes xanthine to superoxide and uric acid. The superoxide formed combines with I.N.T2-(4-iodophenyl)-3-(-4-nitrophenol)5-phenyltetrazolium chloride to produce a

red coloured formazan dye. The superoxide dismutase in the sample inhibits this reaction and so the decrease in intensity is measured .



Reagent composition

Mixed Substrate	–	Xanthine(0.05mmol/l)
		I.N.T.(0.025mmol/l)
Buffer	-	CAPs
		EDTA(0.94mmol/l)
Xanthine Oxidase	-	80U/l
Standard	-	4.4U/L
Sample Diluent	-	Phosphate Buffer pH7.00(.01mol/l)

Reagent preparation

1. **Mixed Substrate (R1)**

The contents of one vial containing Mixed Substrate R1 is reconstituted with 20 ml of Buffer R2..

2. Buffer (R2)

Ready for use.

3. Xanthine Oxidase (R3)

one vial of R3 was reconstituted with 10ml of distilled H_2O.

4. Standards (R4)

One vial of standard R4 was reconstituted with 10ml of distilled water.

Various standers dilutions were prepared with sample diluent (R5). The following dilutions were made of standard S6 to produce a standard curve.

Volume of Standard Solution	**Volume of Sample Diluent**
S6 Undiluted Standard	
S5 5ml OF S6	5ml
S4 5ml OF S5	5ml
S3 5ml OF S4	5ml
S2 3ml OF S3	6ml

S1 = Sample Diluent

Procedure ;

25 µL of diluted sample(100 times diluted with sample diluent) was mixed with 850 µL of mixed substrate(xanthine and I.N.T) and 125µL of xanthine oxidase was added. Mixed and incubated for 30 seconds at 37 degree Celsius ,then the first reading was taken at 505nm. Read again after 1,2 and 3 minutes.

Calculation

The mean absorbance change per minute was determined.

$$\text{Activity} = \frac{\text{Asample/min}}{\text{As1/min}} \times 100$$

$$\% \text{ inhibition} = 100 - \text{activity}$$

The percentage inhibition for each standard was plotted against Log10 (standard conc. In SOD units/ml).

A graph was drawn with percentage of inhibition of standards in one axis and log of superoxide dismutase level in other axis.

The percentage inhibition of sample was used to obtain units of SOD from standard curve.

SOD units/ml of whole blood = Sod units /ml from standard curve x dilution factor.

SOD activities are expressed as units per ml.

Reference range:

164-240U/ml.

STATISTICAL ANALYSIS

1. Age, BMI, fasting lipid profile and plasma glucose were compared between the 3 study groups by ANOVA.

2. Duration of diabetes, hypertension, plasma glucose, plasma lipid profile were compared between group 1 (diabetes with CVD) and group 2(diabetes without CVD) by t test.

3. Smoking, alcoholism and gender between 3 groups was compared by chi square test.

4. SOD Genotype frequency (TT, CT, and CC) distribution between cases and controls were compared with Chi square test.

5. Odds ratio was calculated for SOD genotype distribution in the study population.

6. Allele (T, C) frequencies were calculated by allele counting.

7. SOD activity was compared between the study groups by ANOVA.

8. SOD activity for MnSOD genotypes were compared by ANOVA.

RESULTS

MASTER CHART

Table -1 Lipid levels, BMI, genotype of Diabetic Patients with Cardiovascular Disease.

SI NO	AGE (YR)	SEX	ECG	Duration of DM	HYT	SMK	ALC	WT (KG)	HT (m)	BMI Kg/m2	Fasting plasma glucose(mg/dl)	CHOL (mg/dl)	TGL (mg/dl)	HDL (mg/dl)	LDL (mg/dl)	SOD activity(U/L)	SOD2 genotype.
1	42	M	IWMI	5	Yes	Yes	yes	70	1.63	26.35	135	257	170	47	176	47	TT
2	40	M	IWMI	6	No	No	yes	76	1.69	26.6	146	156	90	50	88	96	CT
3	60	F	AWMI	8	Yes	No	No	66	1.51	28.95	124	163	110	65	76	106	CT
4	75	M	IWMI	10	Yes	No	No	72	1.6	28.13	210	157	103	53.6	82.8	162	CT
5	55	M	AWMI	6	No	yes	yes	78	1.6	30.47	154	245	243	60	136.4	53	TT
6	56	M	AWMI	5	No	No	No	76	1.7	26.3	131	167	135	70	70	62	TT
7	47	M	AWMI	5	No	No	No	78	1.6	30.47	135	170	134	72	71.2	59	TT
8	55	F	AWMI	5	No	No	No	65	1.53	27.8	170	155	101	69	65.8	53	TT
9	46	M	IWMI	6	Yes	Yes	No	75	1.65	27.6	181	156	145	55	72	109	CT
10	45	M	AWMI	5	Yes	Yes	yes	81	1.63	30.45	189	167	132	67	73.6	62	TT
11	47	M	IWMI	4	No	Yes	yes	79	1.59	31.35	208	175	146	64	81.8	47	TT
12	50	F	AWMI	6	No	No	No	63	1.54	26.58	285	256	197	45	171.6	115	CT
13	51	F	IWMI	7	Yes	No	No	65	1.58	26	156	287	231	46	194.8	67	TT
14	47	M	AWMI	4	Yes	Yes	No	67	1.66	24.36	175	161	156	76	53.8	67	TT
15	48	M	AWMI	4	Yes	Yes	yes	74	1.67	26.52	121	166	142	65	72.6	46	TT
16	50	M	IWMI	5	Yes	Yes	yes	76	1.64	28.25	110	155	136	78	49.8	96	TT
17	45	F	AWMI	6	No	No	No	66	1.54	27.85	95	176	145	65	82	162	CC
18	47	F	AWMI	4	No	No	No	57	1.56	23.45	128	202	199	48	114.2	56	TT
19	55	M	IWMI	7	Yes	Yes	No	78	1.64	29	94	209	203	54	114.4	152	CT
20	56	M	IWMI	6	No	No	yes	73	1.6	28.51	226	170	145	78	63	56	TT
21	48	F	IWMI	8	Yes	No	No	58	1.53	24.78	157	189	190	75	76	59	TT
22	45	F	AWMI	4	Yes	No	No	59	1.55	24.58	151	176	145	46	101	68	TT
23	57	M	AWMI	5	No	Yes	yes	64	1.7	22.14	142	223	190	45	140	71	TT
24	59	M	AWMI	5	Yes	Yes	yes	81	1.71	27.7	152	245	204	68	136.2	121	CT
25	64	M	AWMI	4	Yes	Yes	No	71	1.64	26.4	145	256	232	69	140.6	75	TT
26	65	F	AWMI	5	No	No	No	64	1.6	25	156	188	178	52	100.4	81	TT
27	57	F	AWMI	6	No	No	No	58	1.53	24.8	167	156	145	56	71	100	CT
28	55	M	IWMI	4	No	Yes	No	82	1.55	34.1	158	158	134	67	64.2	87	TT
29	56	F	IWMI	5	Yes	No	No	59	1.54	24.9	137	261	234	48	166.2	143	CT
30	57	F	AWMI	4	Yes	No	No	66	1.57	26.5	145	186	165	53	100	90	TT

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Table -2 Lipid levels, BMI, genotype of Diabetic Patients without Cardiovascular Disease.

SI NO	Age (YR	SEX	ECG	Duration of diabetes (YR)	HYT	SMK	ALC	WT (kg)	HT (m)	BMI kg/m^2	Fasting plasma glucose (mg/dl)	CHOL (mg/dl)	TGL(mg/dl)	HDL (mg/dl)	LDL(mg/dl)	SOD activity(U/L)	SOD 2 Genotype
1	40	M	N	4	Yes	No	No	75	1.63	28.3	156	286	245	66	171	75	TT
2	55	M	N	5	Yes	Yes	No	64	1.66	23.27	124	210	90	68	124	71	TT
3	45	M	N	4	Yes	Yes	yes	78	1.51	34.2	174	170	110	72	76	122	CT
4	65	F	N	4	No	No	No	64	1.56	26.33	97	223	103	75	127.4	96	CT
5	66	F	N	5	Yes	No	No	76	1.53	32.4	113	245	231	57	141.8	97	TT
6	70	M	N	5	Yes	Yes	yes	65	1.7	22.49	123	256	135	45	184	162	CT
7	65	M	N	6	No	Yes	yes	78	1.65	28.6	228	170	203	78	51.4	206	CC
8	68	M	N	8	No	No	No	79	1.66	28.7	96	155	101	67	67.8	97	TT
9	45	F	N	4	No	No	No	57	1.6	22.2	146	156	145	64	63	143	CT
10	56	M	N	5	No	No	yes	81	1.65	29.7	154	165	132	73	65.6	121	CT
11	47	M	N	4	Yes	Yes	yes	79	1.67	28.4	152	175	234	46	82.2	100	TT
12	42	M	N	5	No	Yes	No	78	1.54	32.9	155	175	197	68	67.6	100	TT
13	66	M	N	6	Yes	Yes	yes	65	1.58	26.1	143	155	231	49	59.8	103	TT
14	65	F	N	6	No	No	No	66	1.58	26.5	157	256	156	67	157.8	200	CC
15	59	M	N	6	No	No	No	74	1.67	26.6	168	189	142	78	82.6	115	CT
16	64	F	N	4	Yes	No	No	58	1.64	20.1	139	155	136	78	49.8	175	CC
17	57	M	N	5	No	Yes	yes	66	1.64	24.6	157	176	145	48	99	131	CT
18	55	M	N	4	No	No	No	57	1.58	22.89	142	202	199	50	112.2	100	TT
19	56	F	N	7	Yes	No	No	56	1.55	23.4	144	209	203	65	103.4	106	TT
20	56	F	N	6	No	No	No	62	1.6	24.2	126	170	145	54	87	162	CC
21	45	M	N	5	Yes	Yes	yes	76	1.71	26	133	189	235	48	94	121	CT
22	48	M	N	6	No	Yes	No	78	1.68	27.6	212	176	145	60	87	115	CT
23	55	M	N	4	Y	N	y	77	1.7	26.6	134	257	190	71	148	169	CC

					es	o	es										
24	56	M	N	5	Yes	No	yes	81	1.71	27.7	137	153	179	73	44.2	106	TT
25	47	F	N	4	Yes	No	No	59	1.54	24.8	175	170	197	72	58.6	162	CT
26	55	F	N	6	Yes	No	No	64	1.6	25	172	245	178	57	152.4	169	CC
27	47	M	N	5	No	No	No	58	1.6	22.6	183	157	145	68	60	115	CT
28	54	F	N	10	No	No	No	62	1.55	25.8	187	158	134	64	67.2	106	TT
29	56	M	N	8	No	Yes	No	59	1.68	20.9	258	158	146	50	78.8	143	CT
30	53	F	N	5	Yes	No	No	57	1.57	23.1	273	186	165	53	100	143	CT

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Table -3 Lipid levels, BMI, genotype of controls

SI NO	Age (YR	SEX	ECG	Duration of diabetes (YR)	HYT	SMK	ALC	WT (kg)	HT (m)	BMI kg/m^2	Fasting plasma glucose (mg/dl)	CHOL (mg/dl)	TGL(mg/dl)	HDL (mg/dl)	LDL(mg/dl)	SOD activity(U/L)	SOD 2 Genotype
1	40	M	N	4	Yes	No	No	75	1.63	28.3	156	286	245	66	171	75	TT
2	55	M	N	5	Yes	Yes	No	64	1.66	23.27	124	210	90	68	124	71	TT
3	45	M	N	4	Yes	Yes	yes	78	1.51	34.2	174	170	110	72	76	122	CT
4	65	F	N	4	No	No	No	64	1.56	26.33	97	223	103	75	127.4	96	CT
5	66	F	N	5	Yes	No	No	76	1.53	32.4	113	245	231	57	141.8	97	TT
6	70	M	N	5	Yes	Yes	yes	65	1.7	22.49	123	256	135	45	184	162	CT
7	65	M	N	6	No	Yes	yes	78	1.65	28.6	228	170	203	78	51.4	206	CC
8	68	M	N	8	No	No	No	79	1.66	28.7	96	155	101	67	67.8	97	TT
9	45	F	N	4	No	No	No	57	1.6	22.2	146	156	145	64	63	143	CT
10	56	M	N	5	No	No	yes	81	1.65	29.7	154	165	132	73	65.6	121	CT
11	47	M	N	4	Yes	Yes	yes	79	1.67	28.4	152	175	234	46	82.2	100	TT
12	42	M	N	5	No	Yes	No	78	1.54	32.9	155	175	197	68	67.6	100	TT
13	66	M	N	6	Ye	Ye	Ye	65	1.58	26.1	143	155	231	49	59.8	103	TT

					s	s	s										
14	65	F	N	6	No	No	No	66	1.58	26.5	157	256	156	67	157.8	200	CC
15	59	M	N	6	No	No	No	74	1.67	26.6	168	189	142	78	82.6	115	CT
16	64	F	N	4	Yes	No	No	58	1.64	20.1	139	155	136	78	49.8	175	CC
17	57	M	N	5	No	Yes	yes	66	1.64	24.6	157	176	145	48	99	131	CT
18	55	M	N	4	No	No	No	57	1.58	22.89	142	202	199	50	112.2	100	TT
19	56	F	N	7	Yes	No	No	56	1.55	23.4	144	209	203	65	103.4	106	TT
20	56	F	N	6	No	No	No	62	1.6	24.2	126	170	145	54	87	162	CC
21	45	M	N	5	Yes	Yes	yes	76	1.71	26	133	189	235	48	94	121	CT
22	48	M	N	6	No	Yes	No	78	1.68	27.6	212	176	145	60	87	115	CT
23	55	M	N	4	Yes	No	yes	77	1.7	26.6	134	257	190	71	148	169	CC
24	56	M	N	5	Yes	No	yes	81	1.71	27.7	137	153	179	73	44.2	106	TT
25	47	F	N	4	Yes	No	No	59	1.54	24.8	175	170	197	72	58.6	162	CT
26	55	F	N	6	Yes	No	No	64	1.6	25	172	245	178	57	152.4	169	CC
27	47	M	N	5	No	No	No	58	1.6	22.6	183	157	145	68	60	115	CT
28	54	F	N	10	No	No	No	62	1.55	25.8	187	158	134	64	67.2	106	TT
29	56	M	N	8	No	Yes	No	59	1.68	20.9	258	158	146	50	78.8	143	CT
30	53	F	N	5	Yes	No	No	57	1.57	23.1	273	186	165	53	100	143	CT

Table 1:

Table-1
Comparison of parameters in Cases and Control

Variables	CASES		Controls Group 3	P-Value	Results
	Group1 DM with CVD	Group 2 DM without CVD			
Age (years)	52.67±7.586	55.27±8.263	55.27±8.103	**0.351**	NS
BMI	27.19±2.557	26.06±3.424	26.24±2.638	**0.273**	NS.

Fasting plasma glucose (mg/dL)	156.10±39.270	158.60±41.199	91.27±4.160	**0.000**	S
Cholesterol (mg/dL)	192.93±40.56	191.57±38.421	152.10±11.158	**0.000**	S
TGL (mg/dL)	162.67±41.7	166.57±43.602	126.87±22.685	**0.000**	S
HDL (mg/dL)	60.22 ±10.854	62.8±10.545	63.367±9.661	**0.459**	NS
LDL (mg/dL)	100.18±39.865	95.45±38.846	63.36 ±12.192	**0.000**	S
Duration of DM (years)	5.47±1.432	5.37±1.426		**0.787**	NS
Hypertension	16(51.6%)	15(48.4%)		**0.796**	NS
Sex-Male	18(32.1%)	19(33.9%)	19(33.9%)	**0.954**	NS
Sex-Female	12(35.3%)	11(32.4%)	11(32.4%)	**0.954**	NS
Smoking	13(37.1%)	11(31.4%)	11(31.4%)	**0.829**	NS
Alcoholism	10(33.3%)	10(33.3%)	10(33.3%)	**1.000**	NS

Age, BMI, Fasting lipid profile and plasma glucose were compared between the 3 study groups by ANOVA. Male and female sex, Smoking, alcoholism between 3 groups was compared by chi square test.

There is no significant difference between confounding factors like age, BMI, male sex, smoking, alcoholism, HDL cholesterol between groups.

There is significant difference in plasma glucose, triglycerides, total cholesterol, LDL-cholesterol between cases and controls.

Table-2

Table-2
Comparison of parameters between group1A and group 1B

Variables	CASES		P-Value	Results
	Group1A DM with CVD	Group 1B DM without CVD		
Fasting plasma glucose (mg/dL)	156.10±39.270	158.60±41.199	**0.810**	NS
Cholesterol (mg/dL)	192.93±40.56	191.57±38.421	**0.893**	NS
TGL (mg/dL)	162.67±41.7	166.57±43.602	**0.724**	NS
HDL (mg/dL)	60.22 ±10.854	62.8±10.545	**0.354**	NS
LDL(mg/dL)	100.18±39.865	95.45±38.846	**0.643**	NS
Duration of DM(years)	5.47±1.432	5.37±1.426	**0.787**	NS
Hypertension	16(51.6%)	15(48.4%)	**0.796**	NS

Duration of diabetes, hypertension, plasma glucose, plasma lipid profile were compared between group 1 (diabetes with CVD) and group 2(diabetes without CVD) by t test.

There is no significant difference in duration of diabetes, hypertension, plasma glucose, plasma lipid profile between group 1 (diabetes with CVD) and group 2(diabetes without CVD).

From this we find that there is no significant difference in confounding factors like age, gender, BMI, smoking, alcoholism, duration of diabetes, hypertension, fasting plasma glucose, lipid profile between diabetic patients with cardiovascular complications and diabetic patients without cardiovascular complications. As all confounding factors are matched , there is no need to perform logistic regression analysis.

Table 3 and **table 4**

TABLE – 3
SOD 2 genotype distribution in study population

Genotype	Group1- DM with CVD 30	Group2 DM without CVD 30	Group 3 Controls 30	P value
TT	20(66.7%)	11(36.7%)	7(23.3%)	**0.006-S**
CT	9(30.0%)	13(43.3%)	14(46.7%)	
CC	1(3.3%)	6(20.0%)	9(30.0%)	

Table -4
Allele frequency and distribution of T+ allele in Diabetic CVD cases and controls

Allele	Group 1-Diabetic CVD Cases	Controls	P Value
T+	29(97%)	21(70%)	P value=0.015
T-	1(3%)	9(30%)	

Shows the genotype distribution and allele frequencies of SOD2 gene in Group1 (type 2 diabetes with CVD), Group 2 (diabetes without CVD) and controls.

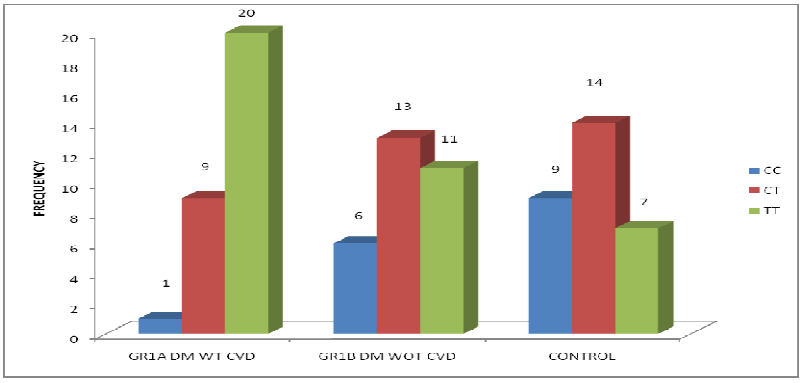
TT genotype is more frequently distributed among diabetics with CVD 20(66.7%) compared to diabetics without CVD 11(36.7%) and controls 1(3.3%). The difference in the frequency of TT genotype was found to be significant between diabetic CVD cases and other two groups as indicated by the P value (0.006).

- CT and CC genotypes are distributed more in the diabetics without CVD and in controls when compared to diabetics with CVD population. In short T+ genotype is more common among cases (97%) when compared to controls (70%). P value is 0.015.

SOD2 genotype distribution is in agreement with the Hardy-Weinberg expectations.

Figure 17

Genotype distribution and allele frequency of SOD2 gene



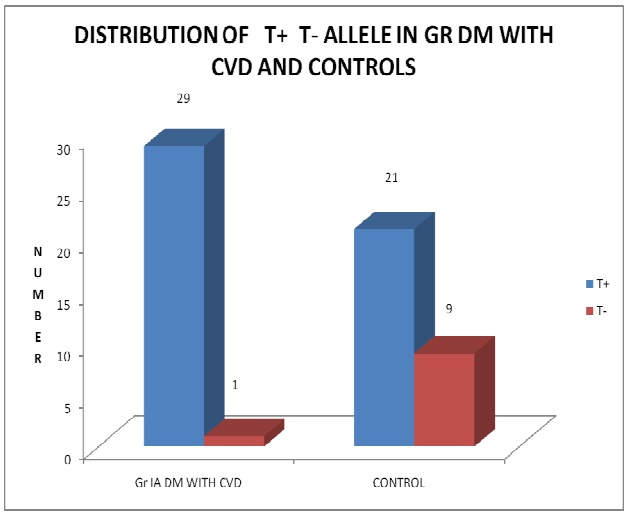

DISTRIBUTION OF T+ T- ALLELE IN GR DM WITH CVD AND CONTROLS
29
1
21
9
30
25
20
15
10
5
0
NUMBER
Gr IA DM WITH CVD
CONTROL
T+
T-

Table 5

Univariate analysis

Allele	Group 1-Diabetic CVD Cases	Controls	Odd's ratio
T+	29(97%)	21(70%)	Odds ratio= 12.45 (0.8-64)
T-	1(3%)	9(30%)	

Shows the Odds ratio calculation on Univariate analysis to evaluate the risk of CVD among T+ genotype individuals.Odds ratio is 12.45 which implies individuals with T+ genotype have 12.45 times increased risk of developing cardiovascular complications.

Table 6

Table-6
SERUM SOD ACTIVITY BETWEEN STUDY GROUPS

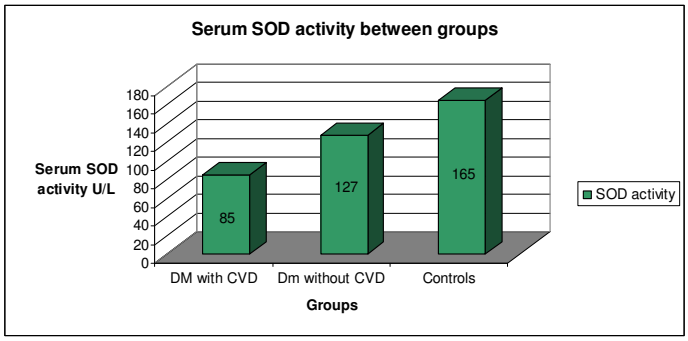
Analyte	Group 1 A DM with CVD	Group 1 B DM without CVD	Group 2 Control	P value
Serum SOD activity U/L	85.60±34.843	127.70±34.771	165.43±43.792	**0.000-S**

Shows serum SOD activity among the three groups.

It is found that serum SOD activity for group 1(diabetics with CVD) was 85 U/L while that of group 2(diabetics without CVD) was 127U/L and that of controls was 165U/L.

P value is 0.000, indicates that the difference is statistically significant.

Our study results indicate that serum SOD activity is reduced in diabetic CVD group compared to other groups.

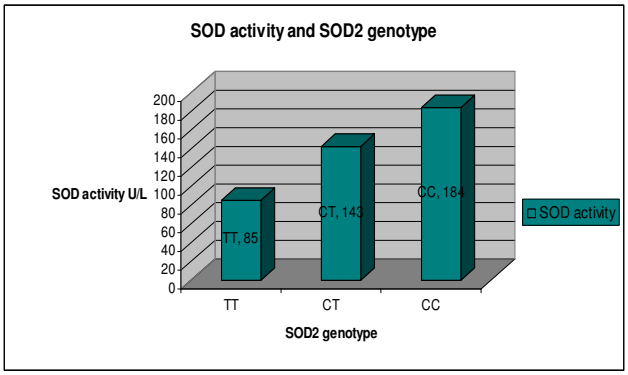


The SOD activity of Group 2 and Control are within the normal range but that of Group 1 (diabetic CVD) is well reduced below lower limit of normal.

Table 7

Table-7
Genotype and Phenotype (SOD activity)

Genotype	SOD activity(U/L)	P value
TT	85.13± 33.228	**0.000-S**
CT	143.92± 31.115	
CC	184.12± 34.415	



Shows the association of SOD genotype with the phenotype (SOD activity)

SOD genotype and its phenotype (SOD activity) were compared.

It is observed that serum SOD activity is reduced in TT genotype.

Reduced (85U/L) level of SOD activity in TT genotype, highest (184U/L) in CC genotype and intermediate(144U/L) in CT genotype .

- P value =0.000 which is highly significant statistically. It implies TT genotype causes reduced SOD activity.

DISCUSSION

In this study we evaluated the association between superoxide dismutase gene 2 Ala16Val polymorphism and cardiovascular complications in diabetes **.** Hyperglycemia and increased free fatty levels in diabetes causes elevated superoxide production in mitochondria which is the major mechanism for cardiovascular complications. This mitochondrial superoxide is normally dismutated by mitochondrial superoxide dismutase. The hypothesis in this study is that polymorphism in mitochondrial targeting sequence of manganese superoxide dismutase gene causing reuced superoxide dismutase activity can accelerate the development of cardiovascular complications.

From our study we found a more frequent association of TT (val/val) genotype in diabetics with cardiovascular complications (66.7%) when compared to controls (3.3%) and type 2 diabetics without cardiovascular Complications (36.7%). The odds ratio for T+ allele was found to be 12 which shows increased risk for cardiovascular complications for those with T allele and p value (0.015) also significant.

This is similar to the study done by Milan Flekac et al, who found positive association between the T allele of the MnSOD gene

polymorphism and diabetic cardiovascular complications in Prague, Czech Republic .

There are no significant differences in confounding factors like age, sex, BMI, smoking, alcoholism, hypertension, plasma triglycerides, LDL-cholesterol between diabetics with cardiovascular complications and diabetics without cardiovascular complications.

In our study we found that serum superoxide dismutase activity was reduced in the diabetics with cardiovascular complications, with the mean value of 85 U/L while that of diabetics without cardiovascular complications group, it was 127U/L and that of controls was 165U/L. The difference was found to be statistically significant (P value=0.000) between diabetics with and without cardiovascular complications and between cases and controls.

We found that individuals with TT (val/val) genotype have lower serum superoxide dismutase activity than those with CC (ala/ala) and CT (ala/val) genotype. This may be due to alteration of conformation of mitochondrial targeting sequence from α helix (present in those with C allele) to β sheets (present in those with T allele) which leads to reduced import of MnSOD enzyme in to mitochondria and degradation by proteosome [114,].. Similar findings were found by Hirori et al and Robert

C.G. Martin et al . This may show that ala 16 val MnSOD gene polymorphism determines the level of mitochondrial superoxide dismutase activity and in turn serum level of superoxide dismutase.

These studies indicate that T (val) allele is associated with oxidative stress where as C(Ala) allele protects against oxidative stress. But contrasting findings have been found in various diseases associated with oxidative stress as follows.

Studies done in American and Finland population had shown positive association of alanine genotype with carcinoma of the breast [87,]. Another study conducted at Taiwan found that individuals with alanine / alanine genotype were more prone for psoriatic arthritis [88]. Positive relation between alalnine genotype and sporadic motor neuron disease was found by study by Van Landeghem et al., 1999a .

A positive association between alanine/alanine genotype and age related macular degeneration was found in study conducted by Kimura et al., 2000,[89] , Shimoda-Matsubayashi et al., 1996 also found increased frequency of ala allele in Parkinson'sdisease [10]. All these diseases which are mainly caused by oxidative stress show increased frequency of C allele except for one study which showed association of T (val) allele with lung cancer [90].

But all the studies of MnSOD gene polymorphism associated with diabetes show positive association of T allele with complications.

Though alanine allele had been found to be associated with cancers in various studies, it had been observed as a protective allele against micro and macrovascular complications in diabetes. For example, a study conducted in USSR by Dimitry A Chistyakov et al [91] found that valine allele was more frequent in diabetic patients with nephropathy than alanine allele. Similar findings were found in studies conducted at Japan and korea[92].

Study done by Jones DA in Caucasian population among diabetic patients found a positive link between reduced level of superoxide dismutase and coronary heart diseae [93]. Sakari Kakko et al found a positive link between this alanine 16 valine manganese superoxide dismutase polymorphism and degree of atherosclerosis in carotid artery.[94]

The present study has shown increased distribution of TT genotype and reduced level of serum superoxide dismutase activity in diabetic patients with cardiovascular complications.

CONCLUSION

The present study was done to find out the SOD2 gene (T>C) substitution polymorphism among diabetics with cardiovascular disease and without cardiovascular disease. 30 cases of diabetics with cardiovascular disease were compared with 30 diabetics without cardiovascular disease.

From our study we found that

1. Type 2 diabetics with cardiovascular disease had a higher frequency of SOD2 TT genotype compared to diabetics without cardiovascular disease and controls.

2. There is a significant difference in TT genotype between diabetics with cardiovascular disease and diabetics without cardiovascular disease.

3. Serum SOD activity is significantly reduced in Diabetics with cardiovascular disease which may be responsible for cardiovascular complications.

4. The level of Serum SOD activity was lowest in TT genotype, and highest in CC genotype and hence TT genotype is strongly associated with cardiovascular disease.

As all the confounding factors are matched, TT genotype is an independent risk factor for the development of cardiovascular complications.

FUTURE PROSPECTS OF THE STUDY

1. Methods to accurately quantify the level of mitochondrial MnSOD activity in leukocytes and correlating them with cardiovascular complications in diabetes can be performed.

2. Trails of SOD mimetic compounds to diabetic patients and their follow up for development of complications may be tried.

Limitations of the study

Since mitochondrial superoxide dismutase(SOD2) contributes only little to the amount of serum level of the enzyme superoxide dismutase, it would have been accurate if mitochondrial superoxide dismutase was measured in mitochondria of leukocytes by differential centrifugation. But as, extraction of mitochondria and its lysis requires centrifugation at very high speed (25,000 rpm) it was not possible to perform in our lab.

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PATIENT CONSENT FORM

Title of the study : "SUSCEPTIBILITY OF DIABETICS WITH SUPEROXIDE DISMUTASE GENE 2 POLYMORPHISM TO VASCULAR COMPLICATIONS "

Name : Date :

Age : OP No :

Sex : Project Patient No :

The details of the study have been provided to me in writing and explained to me in my own language.

I confirm that I have understood the above study and had the opportunity to ask questions.

I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without the medical care that will normally be provided by the hospital being affected.

I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s).

I have been given an information sheet giving details of the study.
I fully consent to participate in the above study.

Signature

ஆராய்ச்சி ஒப்புதல் கடிதம்

ஆராய்ச்சி தலைப்பு: நீரிழிவு பாதிப்புகள் உள்ள நோயாளிகளுக்கு சூப்பராக்சைட் டிச்முடேஸ் மரபணுவின் வேறுபாடு உள்ளதா என்பதை கண்டறிதல்

பெயர் : தேதி :
வயது : புற நோயாளி எண் :
பால் : ஆராய்ச்சி சேர்க்கை எண் :

இந்த ஆராய்ச்சியின் விவரங்களும் அதன் நோக்கங்களும் முழுமையாக எனக்கு தெளிவாக விளக்கப்பட்டது.

எனக்கு விளக்கப்பட்ட விஷயங்களை புரிந்து கொண்டு நான் எனது சம்மதத்தைத் தெரிவிக்கிறேன்.

எனக்கு இரத்த/ சிறுநீர் பரிசோதனை செய்து கொள்ள சம்மதம்.

இந்த ஆராய்ச்சியில் பிறரின் நிர்பந்தமின்றி என் சொந்த விருப்பத்தின் பேரில் தான் பங்கு பெறுகிறேன் மற்றும் நான் இந்த ஆராய்ச்சியிலிருந்து எந்நேரமும் பின்வாங்கலாம் என்பதையும், அதனால் எந்த பாதிப்பும் ஏற்படாது என்பதையும் புரிந்து கொண்டேன்

நான் நீரிழிவு
நோயில் சூப்பராக்சைட்டிச்முடேஸ் மரபணுவின் வேறுபா
டை அறிய மேற்கொள்ளப்படும் இந்த ஆராய்ச்சியின்
விபரங்களைக் கொண்ட தகவல் தாளைப் பெற்றுக்
கொண்டேன்.

இதன் மூலம் எந்த பின்விளைவும் வராது என மருத்துவர் மூலம் தெரிந்து கொண்டு என்னுடைய சுயநினைவுடன் மற்றும் முழு சுதந்திரத்துடன் இந்த மருத்துவ ஆராய்ச்சியில் என்னை சேர்த்துக்கொள்ள சம்மதிக்கிறேன் .

கையொப்பம்

ஆராய்ச்சி தகவல் தாள்.

தங்களது இரத்தம்/ சிறுநீர் இங்கு பெற்றுக் கொள்ளப்பட்டது.

சென்னை அரசு பொது மருத்துவமனைக்கு வரும் நோயாளிகளிடம்இருக்கும் நீரிழிவுநோயில் சூப்பராக்சைட்டிச்முடேஸ் மரபணுவின் வேறுபாடை அறிய மேற்கொள்ளப்படும் ஆராய்ச்சி இங்கு நடைபெற்று வருகின்றது.

நீரிழிவு நோயின் பாதிப்புகளின் காரணங்கள் பல உள்ளன.அவற்றுள் ஒரு மரபணுவின் வேறுபாடு ஒரு காரணமாக இருக்கலாம் என்பதைக் கண்டுபிடிப்பதே இவ்வாரய்ச்சியின் நோக்கமாகும்.

நீங்களும் இந்த ஆராய்ச்சியில் பங்கேற்க நாங்கள் விரும்பிகிறோம்.இந்த ஆராய்ச்சியில் உங்களுடைய இரத்தம்/சிறுநீர் எடுத்து சில சிறப்புப் பரிசோதனைக்கு உட்படுத்தி அதன் தகவல்களை ஆராய்வோம். அதனால் தங்களது நோயின் ஆய்வறிக்கையோ அல்லது சிகிச்சையோ பாதிப்புக்கு உள்ளாகாது என்பதையும் தெரிவித்துக்கொள்கிறோம்.

முடிவுகளை அல்லது கருத்துக்களை வெளியிடும் போதோ அல்லது ஆராய்ச்சியின் போதோ தங்களது பெயரையோ அல்லது அடையாளங்களையோ வெளியிடமாட்டோம் என்பதையும் தெரிவித்துக்கொள்கிறோம்

இந்த ஆராய்ச்சியில் பங்கேற்பது தங்களுடைய விருப்பத்தின் பேரில் தான் இருக்கிறது.மேலும் நீங்கள் எந்நேரமும் இந்த ஆராய்ச்சியிலிருந்து பின்வாங்கலாம் என்பதையும் தெரிவித்துகொள்கிறோம்.

இந்த சிறப்பு பsரிசோதனைகளின் முடிவுகளை ஆராய்ச்சியின் போது அல்லது ஆராய்ச்சியின் முடிவின் போது தங்களுக்கு அறிவிப்போம் என்பதையும் தெரிவித்துக்கொள்கிறோம்.

ஆராய்ச்சியாளர் கையொப்பம் பங்கேற்பாளர் கையொப்பம்

தேதி :

No.	

SUSCEPTIBILITY OF DIABETICS WITH SUPEROXIDE DISMUTASE GENE 2 POLYMORPHISMS TO VASCULAR COMPLICATIONS.

PROFORMA

Name: Age/Sex: IP /OP No:

Address: Phone No:

Ward

Diagnosis:

Presenting complaints: Duration:

Past history:

Chest pain

Stroke

Peripheral neuropathy

Diabetic foot

Vision problems

Renal disease

Personal History:

Smoking

Alcoholism

Tobacco chewing

Menstrual history

Diet history

Family history

Examination :

Vital data :

Heart Rate :

Blood pressure (mean) :

Height :

Weight :

Systemic examination :

CVS

RS

ABDOMEN

CNS

PNS

Impression

Investigations:

Fasting plasma glucose

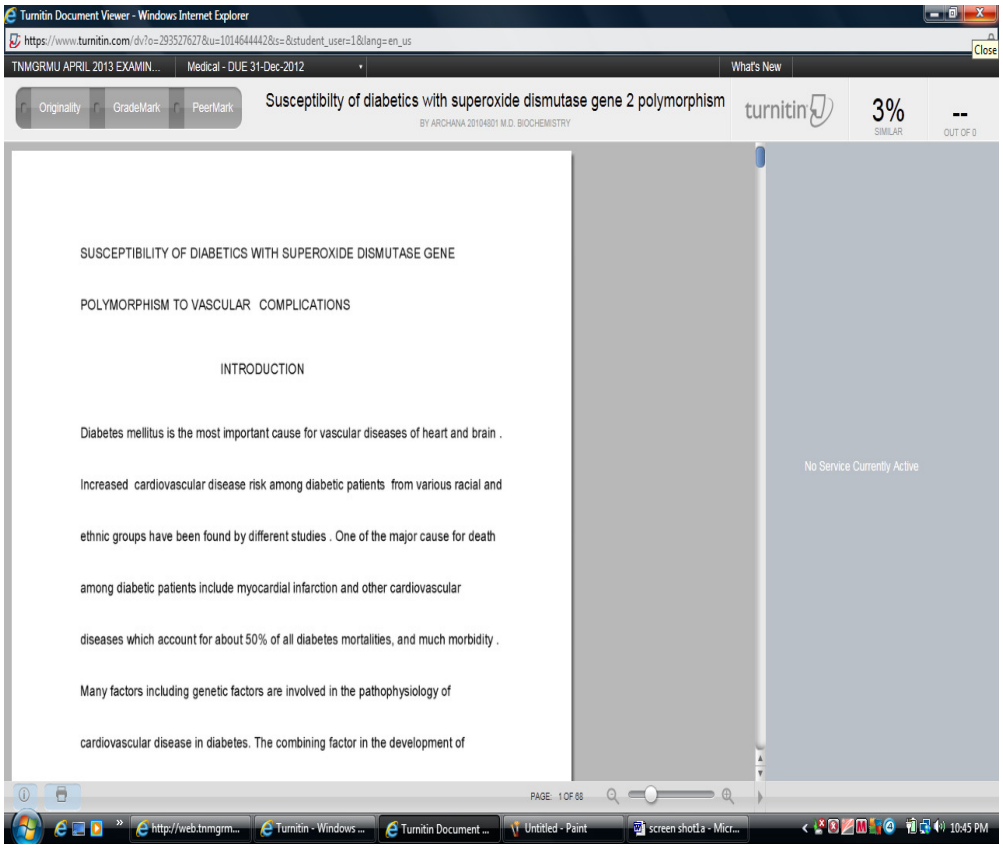
Fasting serum superoxide dismutase level

Fasting lipid profile

Superoxide dismutase gene 2 polymorphism-RFLP PCR,3%agarose gel electrophoresis.

ECG

ANTIPLAGIARYSIM CHECK



INSTITUTIONAL ETHICS COMMITTEE
MADRAS MEDICAL COLLEGE, CHENNAI -3

Telephone No: 04425305301
Fax : 044 25363970

CERTIFICATE OF APPROVAL

To
Dr. M.C. Archana
PG in MD Biochemistry
Madras Medical College, Chennai -3

Dear Dr. M.C. Archana

The Institutional Ethics Committee of Madras Medical College reviewed and discussed your application for approval of the proposal entitled "Susceptibility of diabetics with superoxide dismutase gene 2 polymorphism to vascular complications" No. 27062011.

The following members of Ethics Committee were present in the meeting held on 24.06.2011 conducted at Madras Medical College, Chennai -3.

1.	Prof. S.K. Rajan, MD	-- Chairperson
2.	Prof. V. Kanagasabai MD Dean, Madras Medical College, Chennai-3,	-- Deputy chairman
3.	Prof. A. Sundaram, MD Vice Principal , Madras Medical College, Chennai -3	-- Member Secretary
4.	Prof R. Sathianathan MD	-- Member
5.	Prof R. Nandhini, MD Director, Institute of Pharmacology, MMC, Ch-3	-- Member
6.	Prof. Geetha Subramanian MD DM Prof & Head , Dept. of Cardiology, MMC, Ch-3	-- Member
7.	Prof. Pregna B. Dolia MD Director , Institute of Biochemistry, MMC, Ch-3	-- Member
8.	Prof. C. Rajendiran .MD Director , Institute of Internal Medicine, MMC, Ch-3	-- Member
9.	Thiru. A. Ulaganathan Administrative Officer, MMC, Chennai -3	-- Layperson
10.	Thiru. S. Govindasamy . BA.BL	-- Lawyer
11.	Tmt. Arnold Soulina	-- Social Scientist

We approve the proposal to be conducted in its presented form

Sd /. Chairman & Other Members

The Institutional Ethics Committee expects to be informed about the progress of the study, any SAE occurring in the course of the study, any changes in the protocol and patient information / informed consent and asks to be provided a copy of the final report

Member Secretary, Ethics Committee